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SCRAPIE PRODUCED EXPERIMENTALLY IN GOATS WITH SPECIAL REFERENCE TO THE CLINICAL SYNDROME

By

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INTRODUCTION

The purpose of this paper is to record observations on experimental scrapie in goats additional to those previously reported (Pattison, Gordon and Millson, 1959; Pattison and Millson, 1960), and to comment in detail on the clinical syndrome.

MATERIALS AND METHODS

These were as described in the papers referred to above.

RESULTS

Clinical Appearance of Experimental Scrapie

The first scrapie-affected goats came under observation in 1955, and since that time 270 affected animals in 55 different experiments have been available for study. A clinical description of scrapie in the goat was given by Pattison *et al.*, (1959) and although that description would still apply to many cases it now requires amplification in the light of experience.

In the first place, it is now recognised that there is extremely wide variation among individual goats in the clinical manifestations of disorder of the central nervous system. Thus, some animals show predominantly symptoms of hyperexcitability, while others are dull throughout the course of the disease; some are terrified of being handled, while others are docile, to the extent of trotting up to be petted; some show marked postural abnormalities from an early stage, while others remain well balanced and active. It is impossible, therefore, to give a composite description of the nervous symptoms observed in scrapie-affected goats because only some of these symptoms will be observed in any one individual.

More striking than the variation in nervous disorder, however, has been the development of the scratching syndrome during passage of the disease through goats. In describing the first scrapie-affected goats observed, Pattison *et al.* (1959) recorded that "several goats showed areas of broken hair produced by scratching with their horns or hind-hooves, and there was a general tendency for the normal 'grooming' nibbling and scratching to be increased, suggesting some degree of skin irritation." This description is consistent with a minimal degree of skin irritation and although this type of case is

still encountered, a far more intense scratching syndrome is now observed in many animals.

The intense scratching syndrome usually appears suddenly and the area of skin to be affected has almost invariably been at the base of the neck anterior to the scapulae (Fig. 1). In a few cases the area primarily involved has been the top of the head (Fig. 2). In many cases scratching of the area with the hind-hooves is so persistent that the skin is broken and bleeding occurs. This period of intense irritation is usually short-lived, and scratching and rubbing of a less frantic type then occur over the rump, along the flanks and on the sides of the neck; if bleeding occurs over the area of skin first affected, a scab usually forms and the area appears to become much less sensitive. Goats showing the early scratching syndrome seldom show obvious derangement of the central nervous system, but as the disease progresses abnormalities of the nervous system gradually develop (as in the 'nervous' form of the disease) and the end-results of unsteady gait and, finally, inability to stand are the same in both forms of the disease. Thus, it is now recognised that the clinical appearance of scrapie-affected goats may or may not include the scratching syndrome. Many animals have been observed that did not scratch throughout the course of the disease (Fig. 3), but many others have shown marked skin irritation at all stages (Fig. 4).

This division of the clinical characteristics of scrapie into two main types has long been recognised among British sheep. Thus, Stockman (1926) wrote that, "In describing the symptoms a usual method has been to divide the clinical picture into stages and to speak of the 'nervous' and 'itching' form of the disease. While that has certain advantages in presenting a picture, it is apt to be misleading if taken too literally; and, as regards attempts to differentiate between a nervous and itching form, it seems quite unjustifiable." He went on to indicate that both nervous and itching symptoms occurred, to a greater or lesser extent, in all affected animals. The inference was that individual variation among sheep determined the dominant clinical picture in individual cases.

From observation of experimentally affected goats, we would suggest a rather different point of view. It appears that the nature of the inoculum used may determine the nature of the clinical syndrome produced. Thus, we have observed that in any one experiment all, or almost all, the animals may show the nervous form, or the scratching form, of the disease, and the number of occasions on which this has occurred makes it difficult to accept that individual susceptibility of the inoculated animal is the only significant factor in determining the kind of disease that occurs. Further, it has been found possible to obtain, by intracerebral goat passage, scrapie brain material that will produce the nervous syndrome in almost all goats inoculated, and other material that will produce the scratching syndrome in almost all goats inoculated. The following figures indicate the extent to which inocula prepared from the brains of

animals showing either the scratching syndrome or the nervous syndrome have reproduced the same syndrome in recent experiments.

Brain inoculum	*No. of animals inoculated*	*No. showing scratching syndrome*	*No. showing nervous syndrome*
Scratching syndrome	21	21	0
Nervous syndrome	51	4	47

It will be noted that the division between the two syndromes is not absolute, but it has been observed that in those animals that develop the nervous form of the disease any scratching that does occur appears later and is less extensive than in the scratching form of the disease. It has also been noted that on the four occasions on which scrapie sheep brain has been inoculated into a total of 25 goats the nervous form of the disease has occurred in 21 cases, and that scratching has not been a feature of the disease. The intense scratching syndrome was first noted after the original sheep material (Pattison *et al.*, 1959) had been passaged intracerebrally five times through goats.

Recovery

The possibility of spontaneous clinical recovery from scrapie was discussed by Stockman (1926) and evidence was given that such recoveries do occasionally occur among sheep. We, too, have noted one such recovery in a naturally-affected Swaledale ewe. This animal was brought to Compton in February 1958, as one of a batch of seven scrapie-affected animals, and the clinical symptoms were characteristic of the disease. In all but one of these animals the disease ran its usual fatal course, but in the exceptional case symptoms of scrapie gradually disappeared, wool grew again over the exfoliated areas and recovery was complete. The recovered animal is still alive and has remained normal in every respect over a period, to date, of about 24 months.

In experimentally affected goats this type of recovery has not been observed. However, very occasionally, remission has been noted in the relentless progress of the disease. Thus, in one animal a period of extensive skin irritation and developing ataxia was followed by a period during which scratching disappeared and hair grew again over the scratched areas; the general condition of the animal improved and recovery appeared likely. After some weeks, however, the characteristic signs of the disease reappeared and the animal was slaughtered on humanitarian grounds approximately eight months after the earliest signs of abnormality had been noted.

On several occasions 'recovery' of a different kind has been noted. In these cases the earliest, indefinite signs of what appeared

to be the scrapie syndrome have passed off after a week or two and one has been left in some doubt as to whether the slight abnormalities noted were in fact those of scrapie. The kind of abnormalities involved have been stamping of the feet, flicking of the tail and slight hyperexcitability. This type of case has invariably occurred in animals inoculated with what might be inocula containing minimal amounts of the scrapie agent, e.g. material subjected to heating or to other processes likely to affect the agent adversely. To this extent it might be postulated that inocula containing very small amounts of the agent may, in some animals, produce transitory signs of scrapie without subsequent development of the typical disease. In the present state of our knowledge, however, it is difficult to be certain that these slight deviations from normality do exist and it is necessary to interpret their meaning with caution.

Scrapie in Goats Inoculated Intracerebrally with Scrapie Goat Brain repeatedly Frozen and Thawed

Wilson, Anderson and Smith (1950) reported that the scrapie agent remained viable in the desiccated state at 0° to 4°C for at least two years, and more recent observations by Stamp, Brotherston, Zlotnik, Mackay and Smith (1959) and Pattison and Millson (1960) indicate that the agent shows an unusual resistance to various adverse physical and chemical treatments. In order to examine the effect of rapid freezing and thawing on the agent, a 10 per cent. w/v suspension of scrapie goat brain in physiological saline was subjected to alternating temperatures of +37°C and approximately −70°C. The low temperature was obtained by using a mixture of dry ice in alcohol. Intracerebral inoculations of castrated male goats about seven months old were made as shown in Fig. 5. The material described as frozen and thawed 12 times was treated over a period of 22 hours, and that frozen and thawed 34 times was treated over a period of 66 hours. The results of the inoculations failed to demonstrate that these treatments had had an adverse effect on the agent.

Scrapie in Goats Inoculated Intracerebrally with Acetone and Ether Extracts of Scrapie Goat Brain

The brain extracts for inoculation were prepared as follows:—200 g. of freeze-dried scrapie goat brain was extracted five times with acetone, using a total of 750 ml. The acetone was distilled from the extract in vacuo and 0.70 g. of the waxy solid remaining was suspended in 10 ml. of saline for injection. The residue after extraction with acetone was dried in vacuo at 30 to 50°C and then extracted four times with ether, using a total of 500 ml. The ether was removed from the extract by distillation in vacuo and 1.16 g. of the waxy solid remaining was suspended in 10 ml. of saline for injection. The residue after ether extraction was dried in vacuo at 30 to 50°C and then extracted twice with M/10 calcium chloride solution, using a total of 300 ml. This extract was filtered and used directly for injection. The residue remaining was washed several times with distilled water by repeated suspension and centrifugation and 1 g. of the washed residue was suspended in 10 ml. of saline for injection.

Goats were inoculated as shown in Fig. 6. The calcium chloride extract was lethal, within 15 minutes, for both goats injected, and these animals are not included in the figure. It will be noted that the untreated brain and the residue produced scrapie after approxi-

Fig. 5

INTRACEREBRAL INOCULATION OF GOATS ON 16.12.58 WITH SCRAPIE GOAT BRAIN SUSPENSION RAPIDLY FROZEN AND THAWED

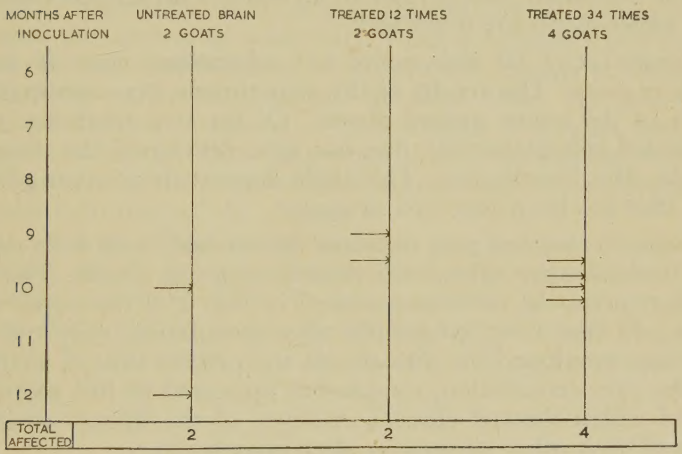
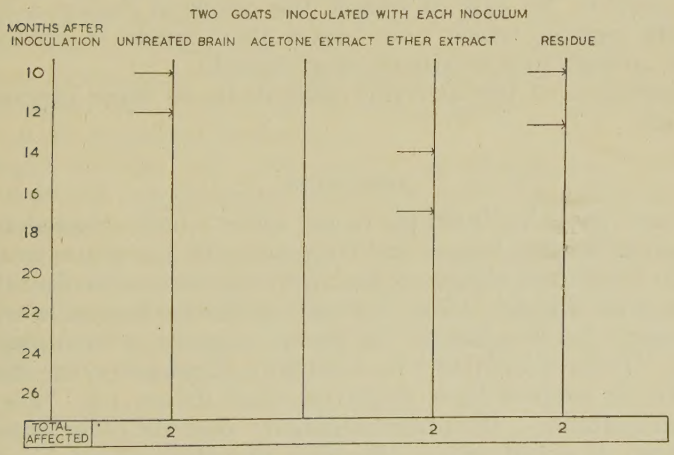


Fig. 6

INTRACEREBRAL INOCULATION OF GOATS ON 17.7.58 WITH VARIOUS SCRAPIE GOAT BRAIN EXTRACTS



mately the same incubation period, but that the appearance of the disease in the animals inoculated with the ether extract was delayed. This result might indicate that the scrapie-producing potential of the ether extract was less than that of either the untreated brain, or

of the residue. The two animals inoculated with the acetone extract have shown no evidence of disease to the time of writing, approximately 24 months after inoculation.

Further Results in Experiments Previously Described

Pattison and Millson (1960) described several experiments that were incomplete at the time of writing, the animals having been under observation for a time believed to be insufficient to cover the possible development of scrapie in all cases. Further observations on these experiments are described:—

Comparison of the intracerebral and subcutaneous routes in producing scrapie in goats. The results of this experiment were summarised in Fig. 1 of the paper quoted above. Of the two surviving animals inoculated subcutaneously one has now developed the disease, 47 months after inoculation. This is the longest incubation period, to date, that has been observed in goats.

Scrapie in sheep and goats inoculated intracerebrally with brain suspension from Swaledale sheep affected with naturally-occurring scrapie. The results of this experiment were summarised in Fig. 2 of the paper quoted above. At that time, 30 months after inoculation, only two of ten goats had developed the disease. At the present time of writing, 43 months after inoculation, scrapie has appeared in five more goats, four of which showed clinical evidence of the disease within two weeks of each other 34 months after inoculation.

Attempts to spread scrapie by direct contact between goats clinically affected with scrapie and non-inoculated goats. Details of the 28 animals included in these experiments were given in Table 1 of the paper above quoted. No case of scrapie has occurred during a further 13-month period, which now brings the periods of observation of these animals to a maximum of 47 months.

Observation of the surviving animals in all these experiments continues.

DISCUSSION

Palmer (1959) has listed the names under which scrapie has been described in Britain, France and Germany, and has drawn attention to the fact that these names are descriptive of the outstanding clinical features of the disease. Thus, "scrapie" in Britain suggests a scratching disease; "la tremblante" in France suggests a trembling syndrome; "Traberkrankheit" in Germany emphasises the trotting gait. Yet in each of these countries other names e.g. "goggles," "prurigo lombaire", "Gnubberkrankheit," describe other features of the disease. It would appear, therefore, that the confusing variety of clinical abnormalities that can be associated with scrapie occur to some extent in all the countries in which the disease is known. But the fact that one clinical picture has appeared to one observer to be so important that a separate name has been invented to describe it,

suggests that in certain flocks, or areas, or even countries, one particular clinical pattern may be more common than all the others.

It appears to us likely that the type of clinical syndrome produced by experimental inoculation will resemble the syndrome exhibited by the animal from which material for inoculation was obtained. The evidence for this conclusion as it applies to goats has been given above, and, in addition, we have observed (unpublished work) that all sheep of 23 different British breeds inoculated with brain suspension from sheep showing the scratching disease developed the scratching syndrome. Thus, it appears likely that the various clinical manifestations of scrapie will reproduce themselves in subsequently affected animals of the same flock. We believe that the basic individual variation in susceptibility to scrapie that exists among sheep may not be so important in determining the dominant clinical picture as the "strain" of the scrapie agent involved in any particular outbreak of the disease.

The presence of the scrapie agent in an ether extract of scrapie goat brain and its apparent absence from an acetone extract of the same brain (as reported above) may provide a useful starting point for those fractionation studies of scrapie-infective tissues that would appear to be indicated as a means of purifying, and eventually identifying, the agent.

CONCLUSIONS

Further observations are recorded on the clinical appearance of goats affected with experimentally-produced scrapie, and comment is made on the occurrence of two clinical syndromes—described as nervous and scratching—that appear to be reproducible by intracerebral inoculation. It is suggested that certain "strains" of the scrapie agent will produce the nervous syndrome, while others will produce the scratching syndrome.

Rapid freezing and thawing of a saline suspension of scrapie goat brain did not apparently affect adversely the infectivity of the scrapie agent.

An ether extract of scrapie goat brain was shown by intracerebral inoculation of goats to contain the scrapie-producing agent, while an acetone extract of the same brain failed to produce the disease when inoculated intracerebrally into goats that have been kept under observation for two years.

ACKNOWLEDGMENT

We are indebted to Mr. R. J. Garner, M.A., M.V.Sc., F.R.C.V.S., A.R.I.C., Compton for kindly preparing the brain extracts treated with acetone and ether.

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[*Received for publication, August 3rd,* 1960]

LEGENDS

Fig. 1. The usual place for scratching to begin in goats showing the "scratching" syndrome.

Fig. 2. A less usual place for scratching to begin in goats showing the "scratching" syndrome.

Fig. 3. Goat affected with the "nervous" syndrome. Note the abnormal posture with the animal leaning to the left. There is no evidence of scratching.

Fig. 4. Goat affected with the "scratching" syndrome. Note the scratched areas over neck, shoulders, back and rump.

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4

OBSERVATIONS ON THE TREATMENT OF *TRYPANOSOMA EVANSI* INFECTION IN CAMELS

By

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INTRODUCTION

Infection with *Trypanosoma evansi* is the only major disease of camels prevalent in the northern Sudan. For some years it has been the practice to treat cases with a standard dose of 5g. Antrypol†, dissolved in 50ml. sterile water and injected intravenously. In certain areas, particularly on the eastern borders of the country, relapses are not uncommon and such cases are re-treated with 2g. Antrycide Methylsulphate‡, dissolved in 20ml. sterile water and injected subcutaneously.

The nomadic tribes quickly recognised the value of Antrycide Methylsulphate when it was introduced and have pressed for its administration in all cases of camel trypanosomiasis. In order to ensure the continued use of Antrypol, which remains an effective drug in the large majority of cases, and to retain the value of Antrycide Methylsulphate as a treatment in reserve, veterinary officers only give the latter drug to camels in which relapses have occurred following one or more injections of Antrypol. In support of this policy, a price differential is applied in the case of privately-owned camels, the charge for treatment with Antrycide Methylsulphate being three times that for Antrypol treatment.

An outbreak of trypanosomiasis occurred amongst working camels belonging to the Public Health Department, Khartoum, during the autumn of 1956. It was brought to the notice of the Research Division when relapses occurred following treatment with Antrypol and the opportunity was then taken to compare the two drugs in routine use with Berenil§, which had recently been introduced as a trypanocidal agent.

MATERIALS AND METHODS

The camels were adult male specimens of *Camelus dromedarius*, their ages ranging from six to over twenty years and weighing from 389 kg. to 559 kg. at the time of their initial treatments. They were picketed in a large open compound when not employed hauling carts. The compound was kept reasonably free from refuse, but biting flies were prevalent and

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† Imperial Chemical (Pharmaceuticals), Ltd. (Suramin, B.P., 1958).

‡ Imperial Chemical (Pharmaceuticals), Ltd., (B. Vet. C., 1953).

§ Farbwerke Hoechst AG. Di-(4-amidinophenyl)-triazene-(N-1:3) diaceturate.

difficult to control since the surrounding area is a heavily populated industrial section of the town.

Diagnosis of trypanosomiasis was effected either by examination of thick blood films or by the mercuric chloride test (Bennett and Kenny, 1928). Films were taken daily, except on Fridays and public holidays, stained by a modification of Field's method (Neave, 1952), then coated with immersion oil and about two hundred fields examined, using the 1/6″ objective. For an organism as large as *T. evansi,* this technique is both effective and rapid.

Antrypol and Antrycide Methylsulphate were administered as described in the Introduction, the former being injected into one of the jugular veins and the latter on the left side at the base of the neck. In certain cases the dosage of Antrypol was raised and the volume injected was then increased proportionately. In accordance with the manufacturer's instructions, Berenil was prepared in a 7 per cent. aqueous solution and injected intramuscularly into the left gluteal region. The recommended dosage rate of 3.5mg. per kg. body weight was used, except for some relapsed cases which were given 7 mg./kg.

RESULTS

The observation period extended from 3rd November, 1956 to 9th June, 1957 (219 days). As can be seen from Fig. 1, numerous re-infections and relapses occurred. The trypanosome showed some degree of resistance to Antrypol and also, possibly, to Berenil although there is insufficient evidence to show whether a dosage rate of 3.5mg./kg. of the latter drug will normally sterilize *T. evansi* infections in the camel.

Results of Treatments

Details of the various treatments given and their results appear in Fig. 1 and are summarized below. It should be emphasized that the term 'initial treatment' refers only to drugs administered during the course of the observation period. As has already been mentioned, these camels were involved in an outbreak of trypanosomiasis when the problem was referred to the laboratory and many had received previous treatments with Antrypol.

Initial treatments with Antrycide Methylsulphate. Four camels received an initial treatment with 2g. Antrycide Methylsulphate and parasites were then not seen in their peripheral circulation for from seventy to one hundred and thirty-eight days, a mean of one hundred and nine days. On re-treatment with the same drug, three remained free to the end of the observation period, a mean of eighty-three days, while trypanosomes were seen in the blood films from the fourth camel after seventy-seven days.

Initial treatments with Antrypol. Since most of these camels had almost certainly received at least one treatment with Antrypol before being referred to the laboratory, only three were given the standard 5g. dose of this drug as an initial treatment. One (488)

Fig. 1

DRUG USED FOR INITIAL TREATMENT IN LABORATORY
CAMEL NO.
1956
1957
NOV.
1st.
DEC.
1st.
JAN.
1st.
FEB.
1st.
MAR.
1st.
APR.
1st.
MAY
1st.
JUN.
ANTRYCIDE METHYLSULPHATE
11
29
465
32
ANTRYPOL
488
34
8
9
10
494
31
5
BERENIL
1
2
15
16
28
30
33
AMS
A5
DIED 13/11
A7·5
A10
T
B7
DIED 7/1
DIED 2/12
B3·5
DIED 28/1
DIED 17/1
A10
TRYPS. SEEN ON 14/3 ONLY
DIED 3/4
DIED 31/3
N5
E
L

LEGENDS—Fig. 1

AMS	2g. Antrycide Methylsulphate
A	5, 7·5 or 10g. Antrypol
B	3·5 or 7mg./kg. Berenil
N	5g. Naganol
Y	Infection syringe-passaged to another camel.
T	Trypanosomes first observed in an artificially infected camel.
IIIIIIIIIIIII	No parasites seen in blood films.
L	Lost trace of camel.
E	End of observation period.

died nine days after this injection, apparently from trypanosomiasis. Another (34) relapsed ten days after treatment and in the third (5) the drug failed to clear the parasites from the peripheral circulation.

Camel 34 then received 2g. Antrycide Methylsulphate and trypanosomes were not seen for fifty days. It developed a low grade infection, parasites being observed on only ten occasions during the hundred and eleven days before it was re-treated with 2g. Antrycide Methylsulphate. Parasites were not seen in the peripheral blood, following this treatment, before the end of the observation period of twenty-six days. Camel 5 was treated with 7 mg./kg. Berenil, after the failure of Antrypol to eliminate the parasitaemia, and trypanosomes were not seen for fifty-seven days. When they re-appeared, the animal was treated with 2g. Antrycide Methylsulphate and remained free to the end of the observation period of ninety-two days.

Blood from camel 34 was transferred to camels 8 and 494, following the first relapse mentioned above, and treatment with 5g. and 7.5g. Antrypol respectively failed to suppress the parasitaemia in either subject. Camel 8 was then treated with 2g. Antrycide Methylsulphate and trypanosomes were not seen until a hundred and seven days later. It was then treated again with the same drug and remained negative to the end of the observation period of twenty-six days. Camel 494 received 10g. Antrypol, but relapsed after eight days. On treatment with 2g. Antrycide Methylsulphate, it remained free until the end of the observation period of one hundred and thirty-eight days.

After the failure of 5g. Antrypol to clear parasites from the peripheral circulation of camel 8, blood from it was inoculated into two further camels, 9 and 10. Both received 7.5g. Antrypol when parasites appeared. No trypanosomes were seen in blood films from

camel 9 for eighteen days after this treatment; when they re-appeared, it received 2g. Antrycide Methylsulphate and remained clear to the end of the observation period of ninety-two days. Parasites were not cleared from the peripheral circulation of camel 10 by doses of 7·5g. and 10g. Antrypol. It was then treated with 7mg./kg. Berenil and parasites were not seen up to the time of death, twenty-five days later.

When 7.5g. Antrypol failed to clear the peripheral circulation of camel 494, infected blood from it was inoculated into camel 31. After a parasitaemia was established, 31 was treated with 10g. Antrypol. Trypanosomes were seen fifty-one days later, but not again during the remaining eighty-seven days of the observation period.

Initial treatments with Berenil. Six camels received 3·5mg./kg. Berenil as their first treatment in the laboratory. Trypanosomes were not seen in the blood of camel 2 up to the time of death, twenty-nine days later. Parasites were seen in the blood films from the remainder (1, 15, 16, 28 and 30) nineteen to sixty days post-treatment, a mean of thirty-eight days.

Subsequently camel 1 died five days after re-treatment with 7mg./kg. Berenil. Camel 15 was treated with 5g. Antrypol and relapsed fourteen days later; it was then treated with 7mg./kg. Berenil and died six days later. Camel 16 was treated with 2g. Antrycide Methylsulphate and remained free for eighty-two days, when trace of it was lost, but it probably died at that time. Camel 28 was re-treated with 3·5mg./kg. Berenil and remained free for eighty-one days. It subsequently received the same treatment a third time and parasites were not seen before it died, seventeen days later. Camel 30 died eleven days after re-treatment with 3·5mg./kg. Berenil.

Following the re-appearance of trypanosomes in the blood of camel 30, after initial treatment with 3·5mg./kg. Berenil, its blood was sub-inoculated into camel 33. This camel had parasites in its peripheral circulation on the next day and was therefore carrying a previous infection. It was treated with 3·5mg./kg. Berenil and parasites were seen in the blood thirty-four days later. It then received 10g. Antrypol and relapsed ten days later. The same treatment was repeated, being administered in error by a veterinary officer who was unaware that this camel was under experiment, and parasites were not seen in the blood for thirty-six days. Finally, it was given 5g. Naganol* and relapsed eleven days afterwards.

Local Reactions

No severe local reactions were noted after treatment with any of these drugs. Camels receiving Antrycide Methyl-sulphate showed a small, localised swelling at the base of the neck, which persisted

* Farbenfabriken Bayer AG., (Suramin, B.P., 1958).

for some months. Those treated with Berenil usually developed a slight swelling at the injection site; this lasted about four or five weeks. Camel 10 developed a marked swelling of the gluteal region three days after treatment with 7mg./kg. Berenil, but this had diminished considerably five days later. There were no untoward local reactions in camels injected with Antrypol.

Deaths

With the exception of camel 5, which survived to the end of the observation period, all camels receiving a single dose of 7 mg./kg. Berenil (1, 10 and 15) died within twenty-five days of this treatment. Of the other six deaths, one (488) occurred, as already mentioned, nine days after initial treatment with 5g. Antrypol, apparently from trypanosomiasis. The other five camels that died had all received 3.5mg./kg. Berenil as their initial treatment. Trace was lost of a sixth camel (16) in this treatment group, but it probably died about the end of May.

Artificial Infections

Six camels (Nos. 8, 9, 10, 31, 33 and 494) were inoculated with infected citrated blood, receiving 10ml. subcutaneously at the base of the neck. As mentioned above, camel 33 had parasites in the peripheral circulation on the following day and was therefore previously infected. Trypanosomes were seen in the blood of the other five animals six to thirteen days after injection, a mean of nine days.

Mercuric Chloride Test Reactions

The mercuric chloride test (Bennett and Kenny, 1928) was carried out weekly on the sera of all camels under observation from immediately before their initial treatments until the end of the twenty-fourth week of the observation period (20th April). The technique of the test is as follows. Blood samples are allowed to stand overnight. One drop of haemoglobin-free serum is dropped cleanly from a Pasteur pipette into 1ml. of a 1:25,000 aqueous solution of mercuric chloride in a small test tube and any haziness developing is graded on a scale ranging from $\pm$ to $+++$. Repeated readings were made on some tests at intervals and no significant differences were observed between readings taken two or three minutes after adding the serum and those made an hour later.

Five naturally infected camels (11, 28, 29, 32 and 465), in which parasites were cleared from the peripheral circulation by their initial treatments, became negative to the mercuric chloride test within six to fifteen weeks, a mean of nine weeks, after these treatments had been given. Subsequently trypanosomes reappeared in the blood of all these camels and their reactions to the test again became positive. After apparently successful re-treatment of three of these camels (11, 28 and 465) their sera again became negative within four to five weeks. These results are summarized in Table 1.

TABLE 1

Camel No.	Days after initial treatment				Days after second treatment	
	Film −	$HgCl_2$ −	Film +	$HgCl_2$ +	Film −	$HgCl_2$ −
11	1	42	115	91	1	35
28	1	44	60	58	1	25
29	1	84	113	121	Not done	
32	1	100	138	121	Not done	
465	1	42	70	84	1	28

Daily mercuric chloride tests were made in the case of the artificially infected camels until a positive reaction developed. Positive readings occurred in ten to fifteen days, a mean of 12·2 days, after infection in five of these camels. Camel 33, in which a previous infection was found to be present, gave a positive reaction with the serum sample taken at the time of injection. This was not known until the following day, when the serum was available for testing.

In an attempt to reduce the subjective element in reading the test, parallel samples were tested in a nephelometer†. It was found that three drops of serum added to 3ml. mercuric chloride gave a satisfactory volume for testing. Bennett (1929) has demonstrated that, providing the quantities of reagents are increased in step, variation of the total volume does not affect the result of the test. Table 2 gives details of the development of the mercuric chloride reaction in three of the artificially infected camels.

TABLE 2

Camel No.	Reading of test	Days after artificial infection								
		7	8	9	10	11	12	13	14	15
9	Visual	−	−	−	not	±	±	±	+	++
	Neph.	15	17	19	done	29	28	31	40	43
10	Visual	−	−	±	not	+	+	+	++	++
	Neph.	20	20	24	done	38	47	42	50	74
31	Visual	not		−	−	−	not	−	−	+++
	Neph.		done	20	16	18	done	18	18	62

Nephelometer values are arbitrary figures read from the scale after setting the instrument at 10-11 with a control tube containing 3 ml. 1:25,000 mercuric chloride solution.

† Evans Electroselenium, Ltd., Halstead, Essex, U.K.

DISCUSSION

In conditions where opportunities exist for re-infection with trypanosomes, it is difficult to distinguish relapses from re-infections. Bennett (1933) states that examination of single wet blood films misses eighty per cent. of infections in camels. Daily examination of stained films probably reduces substantially this proportion of failures, but the method cannot be regarded as very accurate. The observations made in the course of this outbreak confirm the findings by Bennett (1929) on the value of the mercuric chloride test as a reliable diagnostic technique. When the simple basic materials have been prepared, it has the advantage of being considerably less time-consuming than blood film examination. Since a case has not been encountered in which positive blood films continued to occur while the mercuric chloride test became negative, it seems probable that a negative reaction implies freedom from infection. Further experimental proof is required on this point but, if the assumption is correct, the test is of particular value in differentiating relapsed cases from re-infections.

From the results of the treatments given, it is apparent that the strain of *T. evansi* had developed a considerable degree of resistance to Antrypol, but was still susceptible to Antrycide Methylsulphate in the majority, if not in all, cases. Since the trypanosomes were Antrypol-resistant, it is impossible to judge whether 3·5mg./kg. Berenil, the manufacturer's recommended dosage rate, would be an effective treatment for susceptible strains of *T. evansi* in camels. However, there can be no doubt that a single injection of 7 mg./kg. Berenil is highly toxic to adult camels.

If faced with an outbreak of this type in the field, injection of all the animals at risk with a single dose of 2g. Antrycide Methylsulphate, coupled with measures to reduce the incidence of biting flies, could reasonably be expected to eliminate the infection.

CONCLUSIONS

The results of various treatments of camels involved in an outbreak of *T. evansi* infection are recorded. The strain of trypanosome was resistant to 5g. Antrypol, but susceptible to 2g. Antrycide Methylsulphate. Berenil (3·5mg./kg.) was ineffective as a treatment, possibly as a result of cross-resistance developing after treatment with Antrypol. Berenil (7mg./kg.) proved highly toxic to adult camels. The value of the mercuric chloride test in observing the course of *T. evansi* infection in camels is confirmed.

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INFECTION OF LABORATORY ANIMALS WITH *MYCOBACTERIUM JOHNEI*

I—Infection in Swiss White Mice and its Modification by Suramin and Cortisone

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INTRODUCTION

Research on Johne's disease has long been hampered by the lack of a suitable laboratory animal as an experimental host. It was for long believed that no laboratory animal was susceptible to infection, but more recently several workers have produced specific lesions in at least a proportion of inoculated animals; it appeared in these cases that animals should be infected in early life and that a long period of incubation was to be expected.

Studies have been made in rabbits by Alikaeva (1940) and Rankin (1958), and in voles by Levi (1950). In mice, Francis (1943) using a repeated dose of organisms, obtained intestinal lesions in a proportion of the mice he examined and believed that the infection was most notable in the liver in the early stages. Lominski, Cameron and Roberts (1956) extended these findings, using Swiss white mice, and found that, provided young mice were inoculated with an adequate dose of organisms by the intravenous or intraperitoneal route, and examination continued for 7 to 20 months, intestinal infection was established in a high proportion of cases; these authors noted that faecal excretion accounted for many more bacilli than were originally inoculated, confirming that multiplication of the organisms took place to constitute a true infection. Ford (1957) recovered viable organisms from the livers and spleens of mice up to a period of forty-two weeks after infection, but could not demonstrate intestinal lesions; he further noted that administration of cortisone did not facilitate the appearance or persistence of *M. johnei* in the mice, but rather assisted the host to eliminate the organism more rapidly than when the organism is injected alone.

The present investigation was designed to observe *M. johnei* infection in mice, to ascertain the constancy of the early liver and spleen infections, and to detect any modification of the infection by administration of the drugs cortisone and suramin. The maximum time of observation of infected mice was limited to one year, since it is considered that any period in excess of this would greatly limit the use of mice as general experimental animals for Johne's disease. Samples of mice were killed at three months, six months and one year after infection, and observations were concentrated on the changes in livers, spleens and intestines.

MATERIALS AND METHODS

Mice. The mice were of the Swiss white strain, maintained as a closed colony at this Field Station for several years. They were used, newly weaned, when three or four weeks old.

*Suramin**. The drug was dissolved in sterile saline so that a dose of 1 mg. in 0·1 ml. could be inoculated subcutaneously.

Cortisone. The drug†, in the form of cortisone acetate was diluted in sterile saline to give the required dose in 0·1 ml. and was inoculated subcutaneously.

Cultural methods. The method of isolation and culture was that used by Taylor (1950) i.e. prior treatment with oxalic acid and subsequent culture on Taylor's modification of Finlayson's medium. All cultures were incubated at 38°C and were examined periodically for three months before being regarded as negative. The methods were qualitative and were intended to give information regarding the presence of viable bacilli.

Strain of M. johnei. The strain used throughout was originally isolated from a case of Johne's disease in a cow; on regular occasions this strain has been used to infect cattle from which it has been re-isolated and maintained on Taylor's medium as stock culture. This strain conformed to the cultural characters for *M. johnei* and furthermore was non-pathogenic for adult rabbits and fowls both before inoculating the mice and also on re-isolation from the mice. It can therefore be described as a recently isolated bovine type *M. johnei* strain.

Inocula. Cultures were harvested at six to seven weeks and the moist bacilli weighed and suspended in the necessary amount of saline to constitute the required dose. The dose was administered in a volume of 0·25 ml. by the intraperitoneal route. In the three experiments to be described the inocula were prepared from different subinoculations of the strain described above.

Smear preparations. Liver and spleen impression smears were stained by the Ziehl-Neelsen method and the degree of infection was estimated by counting the numbers of bacilli seen per field, using a 1/12″ oil-immersion lens and x6 eyepiece. Approximately 25 representative fields were examined to calculate the average number per field; 50 fields were examined before a negative classification was made. The results were recorded as follows: − = no bacilli seen; *S* = one bacillus only; 1 + = occasional bacilli on searching; 2 + = single bacilli readily found, or groups on searching; 3 + = groups readily found; 4 + = one group per field; 5 + = more than one group per field. Smear preparations of intestine were first made by impression of the whole of a portion of intestine. This method was shown in the course of the experiments to be unsatisfactory since it revealed infection not only of the mucosa and submucosa but also of the intestinal mesentery or serosa. Later preparations were made by scraping the internal wall of an opened intestine and avoiding contact with the serosa as far as possible.

Histological preparations. These were fixed in formalin and stained by the Ziehl-Neelsen method in all cases, and in some cases also by haematoxylin

* Suramin is the symmetrical 3″ -urea of the sodium salt of 8-(3-benzamido-4-methylbenzamido) naphthalene-1:3:5-trisulphonic acid. It is marketed by Imperial Chemical Industries (Pharmaceuticals) Ltd. as a trypanocide, under the trade-name "Antrypol".

† Obtained from Glaxo Laboratories Limited.

and eosin also. An estimate of the extent of the liver and spleen infections was made by counting the numbers of bacilli seen per microscope field, using a 1/6 lens and x6 eyepiece. Approximately 30 representative fields were examined. The whole preparation was examined before a negative classification was made. The results were recorded as the average number of groups of bacilli/number of fields, e.g. 1/12 signified one group of bacilli for every 12 fields examined, 3/1 signified three groups of bacilli for every field examined. Subsidiary studies and observations during the course of the experiments showed that infection throughout the different parts of a given liver or spleen was reasonably constant and that this method of sampling was efficient in the practical sense; all liver samples were taken from the posterior border of the left lobe and all spleen samples from the ventral end of the spleen.

RESULTS

Course of Infection over a One Year Period, and Effect of Suramin or Cortisone Treatment

A plan of the experiment is shown in Table 1. Three groups each of 50 mice were used; one group was treated with suramin, one was treated with cortisone, and one was left untreated. All three groups were inoculated intraperitoneally with *M. johnei*, three doses namely, 24 mg, 8 mg and 2 mg, being used in each group respectively. The dose regimen for suramin was known to be satisfactory for long-term administration (Rees, 1958)—it was terminated at 8 weeks for practical reasons. The dose regimen for cortisone was selected since preliminary experiments on toxicity had shown that a dose of 0·1 mg. cortisone was suitable for each chosen day of a 4 week period of treatment; it reduced the leucocyte count but did not cause clinical symptoms.

Between two and fifteen days after the *M. johnei* inoculum, a total of eighteen deaths occurred in mice given the 24 mg. dose of *M. johnei*, 6 deaths occurring in the suramin group, 3 deaths in the cortisone group and 9 deaths in the control group; it was concluded that the 24 mg. dose of *M. johnei* was excessive.

Two months after infection the control group (C) weighed approximately 10 per cent more than the suramin group and a proportion of the latter developed an unkempt, hidebound appearance; no other signs of drug toxicity were apparent. At this time one mouse from each group given 24 mg. *M. johnei* was killed. Large numbers of bacilli were seen in the livers and spleens of all three mice; bacilli were also present in the serosa of the intestines, but not in the intestines proper.

Three Months

Three months after infection samples of mice from each group were killed and examined.

Post-mortem appearance. Of the mice given the 24 mg. inoculum, the two in the suramin group showed enlarged livers and spleens;

TABLE 1

PLAN OF EXPERIMENTS

	Group	*No. mice*	*Drug*	*Dose*	*Frequency*	*Course*	M. johnei *inoculum*	*Sampling periods after infection*	*Duration of experiment after infection*
	A	50	Suramin	1 mg	Twice a week	1 week before and 8 weeks after infection	15 mice—24 mg 20 mice— 8 mg 15 mice— 2 mg	3 months, 6 months and 1 year	1 year
	B	50	Cortisone	0·1 mg	Three times a week	1 week before and 4 weeks after infection	,,		
	C	50		Nil			,,		
	A	40	Suramin	1 mg	Three times a week	1 week before and 4 weeks after infection	20 mice— 8 mg 20 mice— 2 mg	10 weeks	10 weeks
	B	40	Cortisone	0·1 mg	Twice a week	,,	,,		
	C	40		Nil			,,		
	D	30	Suramin	1 mg	Three times a week	1 week before and 4 weeks after infection	Nil		
	A	40	Suramin	1 mg	Twice a week	1 week before and 8 weeks after infection	20 mice — 8 mg 20 mice— 2 mg	8 weeks	8 weeks
	B	40	Cortisone	0·1 mg	Three times a week	1 week before and 8 weeks after infection	,,		
	C	40		Nil			,,		
	D	30	Suramin	1 mg	Twice a week	1 week before and 8 weeks after infection	Nil		

the mice of the other groups showed enlarged spleens. The mice of all three groups given the lower doses of *M. johnei* were normal in appearance.

Smears and histology. The results of counts made on smears from and histological preparations of the livers, spleens and intestines stained by the Ziehl-Neelsen method are summarised in Table 2. There was a reasonable degree of correlation between examinations of impression smears and histological preparations. Positive smears of intestines could not be confirmed histologically. In these cases the bacilli seen were in all probability those present in the serosa of the intestine and did not therefore represent true Johne's disease infection, but rather were related directly to the use of the intraperitoneal route of inoculation. At the 24 mg. dose level, liver and spleen examinations

TABLE 2

THREE MONTH SAMPLE

Group	Drug	Inoculum	Mouse	Liver		Spleen		Ileum			Colon		
				Smear	Histo.	Smear	Histo.	Smear	Histo.		Smear	Histo.	
									Intest.	Serosa		Intest.	Serosa
A	Suramin	24 mg	A1	3+	3/1	3+	2/1	−	−	+	−	−	+
			2	5+	3/1	2+	2/1	+	−	++	−	−	++
B	Cortisone	24 mg	B1	3+	1/2	3+	1/2		−	+	+	−	·
			B2	2+	1/6	2+	1/5	+	−	·	−	−	+
C	—	24 mg	C1	2+	1/6	2+	1/10	−	−	·	−	−	·
			2	2+	1/2	1+	1/10	−	−	−	+	−	+
A	Suramin	8 mg	A3	4+	2/1	3+	1/1	−	−	·	−	−	++
			4	3+	2/1	2+	1/2	+	−	++	−	−	·
			5	4+	3/1	2+	1/2	−	−	++	+	−	++
			6	3+	1/2	2+	1/2	−	−	+	−	−	++
			7	3+	2/1	3+	1/1	+	−	·	+	−	·
B	Cortisone	8 mg	B3	2+	1/3	3+	1/30	−	−	·	−	−	·
			4	−	−	−	1/30	−	−	·	−	−	−
			5	−	1/10	S	1/30	−	−	·	−	−	·
			6	−	−	1+	−	−	−	·	−	−	−
			7	S	1/10	−	−	−	−		+	−	·
C	—	8 mg	C3	2+	1/2	2+	1/3	−	−	·	−	−	·
			4	2+	1/2	2+	1/8	−	−	+	−	−	·
			5	2+	1/4	2+	1/5	−	−	·	−	−	++
			6	−	1/5	2+	1/5	S	−	·	−	−	·
			7	1+	1/2	1+	1/20	−	−	·	−	−	·
A	Suramin	2 mg	A8	1+	1/1	1+	1/10	−	−	+	−	−	·
			9	S	1/5	−	−	−	−	·	−	−	−
			10	3+	1/2	1+	1/3	−	−	−	−	−	·
			11	1+	1/3	1+	1/8	−	−	++	−	−	·
			12	2+	1/2	2+	3/1	−	−	+	−	−	·
B	Cortisone	2 mg	B8	−	−	−	−	−	−	·	−	−	·
			9	2+	−	2+	1/10	−	−	−	−	−	·
			10	2+	1/6	2+	1/10	−	−	+	−	−	−
			11	1+	−	1+	1/30	−	−	·	−	−	−
C	—	2 mg	C8	2+	1/6	−	1/10	−	−	·	−	−	++
			9	2+	1/10	−	−	−	−	·	−	−	−
			10	2+	1/2	2+	1/4	−	−	·	−	−	·
			11	−	−	−	−	−	−	·	−	−	−
			12	S	1/4	1+	1/5	−	−	·	−	−	·

The symbols representing intensity of infection, in the livers and spleens, are classified under "Materials and Methods". The symbols representing intensity of infection in the intestines are as follows: ++ = numerous bacilli
+ = few bacilli
− = no bacilli seen
· = not examined

indicated a higher degree of infection in those mice treated with suramin. At the 8 mg. dose level, liver and spleen examinations again indicated a higher degree of infection in the suramin treated group, whereas the cortisone treated group had a lower degree of infection than the other groups. At the 2 mg. dose level the liver histological assessments again indicated a lower degree of infection in the cortisone treated mice. In none of the mice examined was any evidence of infection of the intestine apparent, except in respect of the outer mesentery wall or serosa.

The general histological picture of the affected livers was of a regularly distributed proliferation of macrophage cells of the reticulo-endothelial system. These formed microscopic "granuloma" type collections along the sinusoids and their position appeared consistent with their having originated from cells of the Küpffer type. Large collections of macrophage cells were prominent in the area of the portal tracts, where there was some apparent increase in the numbers of lymphoid cells. There was no evidence of necrosis. In sections stained by the Ziehl-Neelsen method the macrophages contained groups of bacilli, in many cases in such enormous numbers as to suggest that bacillary multiplication had occurred. Figs. 1, 2 and 3 depict these changes in an affected liver. In the spleen as in the liver, there were collections of macrophage type cells containing bacilli, distributed throughout the pulp and in the capsule. No necrosis was observed.

Cultures. The liver and spleen of every mouse were sampled. All but two of the cultures produced luxuriant growths of *M. johnei.* In cultures made from mouse B8, those from the liver were negative and from the spleen only one tube showed a light growth of bacilli; this was the only evidence of infection in this mouse.

Three Months to Six Months

One suramin treated mouse and one control mouse, in the 24 mg. dose group, died; smears indicated infection in the intestines. One control mouse in the 8 mg. dose group died showing infection of liver and spleen only, and one cortisone treated mouse, in the 2 mg. dose group, died but showed no evidence of infection on smear examinations.

Six Months

Post-mortem appearance. In the groups infected with 24 mg. *M. johnei,* both those given suramin had an unkempt appearance and enlarged spleens, one of the three controls had an enlarged spleen, but the cortisone group appeared normal. The remainder of the mice appeared normal except for enlarged spleens in those treated with suramin and infected with 8 mg. *M. johnei.*

Smears and histology. The findings in these groups are summarised in Table 3. At the 24 mg. level, liver examinations again suggested a higher degree of infection in the two suramin treated mice, and intestine examination showed both mice to harbour infection in the colon.

TABLE 3

SIX MONTH SAMPLE

Group	Drug	Inoculum	Mouse	Liver		Spleen		Ileum		Colon	
								Histo.		Histo.	
				Smear	Histo.	Smear	Histo.	Intest.	Serosa	Intest.	Serosa
A	Suramin	24 mg	A13	5+	1/1	2+	1/2	–	·	+	·
			A14	3+	4/1	1+	1/2	–	+	+	+
B	Cortisone	24 mg	B12	S	1/2	1+	1/2	–	+	–	·
			13	2+	1/1	2+	1/2	–	+	–	+
			14	2+	1/1	1+	1/1	–	+	–	·
			15	2+	1/2	1+	1/2	–	+	–	+
C		24 mg	C13	2+	1/1	1+	1/3	–	·	–	·
			14	2+	1/4	2+	1/1	–	·	–	·
			15	2+	1/1	3+	1/1	–	+	·	·
A	Suramin	8 mg	A15	3+	1/10	1+	1/2	–	+	–	+
			16	2+	2/1	1+	1/1	–	+	–	+
			17	3+	2/1	2+	1/7	–	–	–	·
			18	5+	3/1	4+	5/1	++	++	++	++
B	Cortisone	8 mg	B16	1+	1/2	S	1/2	–	–	–	·
			17	2+	1/1	2+	1/4	–	·	–	–
			18	1+	1/2	S	1/50	–	+	–	·
			19	2+	1/3	–	1/5	–	·	–	·
			20	1+	1/1	–	1/5	·	·	·	·
C		8 mg	C16	1+	1/3	–	1/10	–	–	–	+
			17	2+	1/2	–	1/5	–	+	–	·
			18	2+	1/4	2+	1/4	–	·	–	·
			19	2+	1/2	2+	1/5	–	·	–	·
			20	2+	1/1	S	1/1	+	++	+	·
A	Suramin	2 mg.	A19	2+	1/1	2+	2/1	++	+	+	·
			20	3+	1/1	1+	3/1	–	+	–	–
			21	1+	1/2	S	1/1	–	·	–	–
			22	1+	1/1	S	2/1	–	·	–	·
			23	2+	1/3	2+	1/1	–	·	–	+
B	Cortisone	2 mg.	B21	3+	3/1	2+	2/1	–	–	+	+
			22	2+	1/5	1+	1/5	–	·	–	·
			23	2+	1/2	–	–	–	–	–	·
			24	1+	1/1	S	1/2	–	·	·	·
			25	1+	1/5	–	1/50	–	·	–	·
C		2 mg.	C21	2+	1/7	S	1/20	–	·	–	·
			22	1+	1/5	S	1/50	–	–	–	–
			23	2+	1/3	2+	1/20	–	·	–	–
			24	1+	1/5	–	–	–	·	–	–
			25	2+	1/4	–	1/8	–	–	–	·

See Footnote to Table 2.

At the 8 mg. dose level, liver examinations also indicated a higher degree of infection in the suramin group, particularly in the case of one mouse which also showed a massive infection of the spleen and of both ileum and colon. One control mouse also showed intestinal infection. At the 2 mg. dose level, there was little noticeable difference between the three groups; one suramin treated mouse showed infection of both ileum and colon, and one cortisone treated mouse infection of the colon. Taking the intestine samples as a whole, infection was more noticeable in the suramin treated groups.

Cultures. The liver and spleen were sampled from each mouse. *M. johnei* was grown in every case showing that all the mice harboured viable bacilli. No difference between the groups was therefore observed.

Six Months to One Year

Fourteen mice died at intervals during this period. In the suramin group, one mouse given 24 mg. *M. johnei*, showed masses of bacilli in intestinal smears and probably died of Johne's disease. In the cortisone group, two mice given 2 mg. *M. johnei* showed a massive intestinal infection and it is considered that they died of Johne's disease.

One Year (Surviving Mice)

Post-mortem appearances. In the suramin group, all the mice had an unkempt appearance and the sizes of the spleens varied. Two mice showed visibly enlarged and thickened small intestines and were later shown to have intestinal Johne's disease lesions. In the cortisone group the mice had abundant abdominal fat. In the control group no abnormalities were noted except for gross splenic enlargement in two cases.

Smears and histology. The results of examinations of stained preparations are summarised in Table 4 which shows that, except at the 2 mg. level, where the suramin mice had heavier infections, there was little difference in the livers and spleens of the three groups. Most liver infections gave an appearance of resolution, the numbers of organisms per group being reduced in many mice. In some the

TABLE 4

ONE YEAR

Group	Drug	Inoculum	Mouse	Liver		Spleen		Intestines			
				Smear	Histo.	Smear	Histo.	Duodenum	Jejunum	Ileum	Colon
A	Suramin	24 mg	A24	2+	1/2	3+	1/3	–	–	–	–
			A25	2+	1/1	4+	1/1	–	–	–	–
B	Cortisone		B26	1+	1/10	1+	1/20	–	–	–	–
A	Suramin	8 mg.	A26	–	–	S	–	–	–	–	–
			27	1+	1/5	2+	1/7	–	–	–	–
			28	2+	1/30	–	–	–	–	–	–
			29	4+	4/1	3+	5/1	++	++	++	++
			30	S	1+	1/3	–	–	–	–	–
B	Cortisone		B27	2+	–	1+	–	–	–	–	–
			28	1+	1/10	1+	–	–	–	–	–
			29	2+	1/10	2+	–	–	–	–	–
			30	1+	1/30	–	–	–	–	–	–
			31	S	–	1+	–	–	–	–	–
			32	2+	1/30	2+	1/7	–	–	–	–
C			C26	1+	1/4	–	1/25	+	–	–	–
			27	1+	1/4	2+	1/10	–	–	–	–
			28	2+	1/8	1+	1/10	–	–	–	–
			29	1+	1/25	S	–	–			
A	Suramin	2 mg.	A31	5+	3/1	3+	3/1	++	++	++	++
			32	–	1/6	1+	–	–	–	–	–
			33	2+	1/15	S	–	–	–	–	–
			34	1+	1/10	1+	1/6	–	–	–	–
B	Cortisone		B33	–	–	–	–	–	–	–	–
C			C30	–	–	–	–	–	–	–	–
			31	1+	–	–	–	–	–	–	–
			32	1+	–		–	–		–	–
			34	–	1/20	S					

See Footnote to Table 2.

macrophages appeared to be concentrated around the portal tracts. Bacilli were not seen in many of the spleens, particularly those of the 8 mg. cortisone and the 2 mg. control groups. Bacilli were detected in the intestine wall proper of only three mice, two of which were suramin treated animals showing massive infection of the livers and spleens as well as of the four intestinal areas. In the intestines large masses of bacilli were present in both mucosa and submucosa together with epitheliod type cells (Figs. 4, 5 and 6); the epithelial lining appeared intact. One mouse in the control group showed similar but less marked accumulations of *M. johnei* in the duodenum.

Cultures. The organism was isolated from livers and spleens of all the mice killed, with the exception of one cortisone treated mouse in which a positive culture was obtained from the spleen only, this being the only evidence of infection.

Suramin or Cortisone Treatment Ten Weeks after Infection

This experiment was planned to examine further the effect of the drugs at the early stage of infection of the liver and spleen. Three groups, each of 40 mice, as in the first experiment, were inoculated with two doses of *M. johnei* and a fourth group, of 30 mice, was treated with suramin but not infected. As will be seen from Table 1, the dose regimens for the drugs differed from those of the first experiment. The suramin dosage was increased by inoculating three times a week in order to enhance its effects, the group treated with suramin alone acting as a toxicity control, but it became evident that this dose was excessive and it was therefore terminated after four weeks. The cortisone dosage was reduced and was given twice weekly for four weeks. All the surviving mice of the infected groups were killed and examined 10 weeks after infection.

The results of the assessment of liver and spleen infections in the histological preparations stained by the Ziehl-Neelsen method were classified as grades of infection as follows: Grade 1 = a count greater than 4/1; grade 2 = 4/1 — 2/1; grade 3 = 1/1 — 1/2; grade 4 = 1/3 — 1/7; grade 5 = 1/8 — 1/15; grade 6 = 1/16 — 1/32; grade 7 = less than 1/32; grade 8 = negative. Histograms, as shown in Fig. 7, were then drawn to illustrate the numbers of mice in each group (as percentages) for each grade of infection. It will be seen from the liver assessments that although the control mice showed variations in count due to individual susceptibilities, there was nevertheless at each dose level, a noticeable shift to the left in the suramin group (higher counts) and a slight shift to the right (lower counts) in the cortisone group, when compared with the control group. A similar observation was noted from the spleen assessments with a higher number of negative counts in the cortisone treated mice.

Suramin or Cortisone Treatment Eight Weeks after Infection

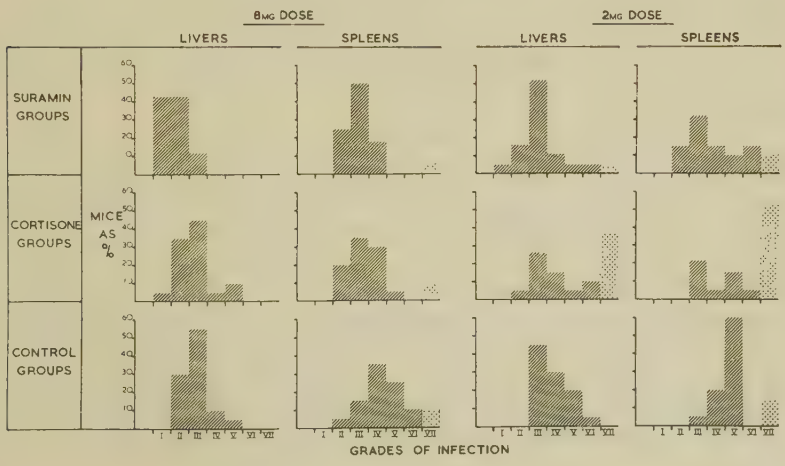
This experiment followed the pattern of the previous trial. The

Fig. 7

suramin dose regimen successfully used in the first instance was adopted, and a toxicity control group was added as a safeguard. A similar cortisone dosage was adopted and was continued for a further four weeks to enhance its effects. All the surviving mice of the infected groups were killed and examined 8 weeks after infection.

The results of the assessment of liver and spleen infections are given in Fig. 8. It will be seen from the liver assessments that the suramin treated groups gave higher counts than the control groups,

Fig. 8



the cortisone group, infected at the 8 mg. dose level, showed no marked difference to its control, while the 2 mg. dose cortisone group showed a higher proportion of negative counts. The spleen assessments, at the 8 mg. dose level, showed increased infection in those treated with suramin and possibly in those treated with cortisone; at the 2 mg. dose level the suramin group gave the highest counts, while the cortisone group gave a large proportion of negative assessments.

DISCUSSION

The results obtained in the control mice of the three experiments showed that infection of the liver and spleen could be detected at a comparatively early stage by the means employed. Although the intensity of infection varied in individual mice, there was a direct correlation between the intensity of infection in the liver and in the spleen of individual mice. Examinations of livers and spleens at intervals from three months up to one year after infection gave no indication that the infections in these organs were progressive, but rather that resolution was taking place. The lesions appeared rather benign, no necrosis being observed, and at the 8 mg. and 2 mg. dose levels did not appear to cause symptoms in spite of the masses of bacilli present in many cases. The impression was that of a protective, clearance process rather than that of an active and progressive lesion.

Six mice of the 38 mice killed in the six month sample (16 per cent), and three mice of the 27 mice killed after one year (11 per cent), showed evidence of intestinal infection on histological examination. Five mice which died during the course of the experiment showed evidence of intestinal infection on smear examinations. It is likely that, had more concentrated examinations of the intestine been made, and had mice been observed for a period greater than one year, more cases would have been diagnosed; nevertheless the incidence of detectable intestinal lesions was so low as to question the value of these Swiss white mice as experimental animals for the study of intestinal lesions of Johne's disease, when used in the manner described. This fact, when considered with the variable intensities of liver or spleen infections in individual mice, indicated that the susceptibility of the mice to infection with *M. johnei* varied greatly between individuals.

The results obtained in all three experiments showed that treatment with suramin resulted in greater numbers of bacilli being counted in the liver and spleen, two to three months after infection. This increase in numbers was most likely due to multiplication of the bacilli after uptake by the liver and spleen. Liver and spleen cultures confirmed the presence of viable bacilli. This enhancement of liver and spleen infection has its parallel in the effect of suramin on the course of B.C.G. infection in mice, as described by Rees and Hart (1956). The latter authors noted that suramin has properties in common with certain non-ionic surface-active agents which also have a "protuberculous" effect. They suggested that within infected

macrophages suramin, or a derivative, modifies the lipid surface of tubercle bacilli in such a way as to make them more resistant to destruction and so results in more active and progressive infection. The present experiments indicate that this activity of suramin is also relevant to *M. johnei*, a point suggested by Rees and Hart in their publication. The results obtained in the first experiment, at the six month sampling, showed that, of the three mice with detectable intestinal infection, two had been suramin treated. At the one year examination a similar result was obtained, the intestinal infections being particularly heavy in the suramin treated mice. There is therefore some evidence that suramin has an enhancing effect on intestinal infection with *M. johnei*. However, the proportion of intestinal infections detected was still very low and it is doubtful if, under circumstances similar to those employed, suramin treatment of similar mice could greatly facilitate their use as general experimental animals for Johne's disease.

The result obtained in cortisone treated mice showed that the infections in the liver and spleen were apparently reduced in intensity two to three months after infection. This is in contrast to the enhancing effect of cortisone on tuberculous infection in the mouse (Hart and Rees, 1950), but is in agreement with the studies of Ford (1957) on *M. johnei* infection in cortisone treated mice. It is likely that the effect of cortisone in the present experiments was due to alteration of the host's reticulo-endothelial system response to infection. The examination of mice for intestinal infection did not show that cortisone had either increased or reduced the proportion of cases of Johne's disease. It was clear that administration of cortisone to mice, under conditions similar to those of the present experiments, would by no means facilitate their use as general experimental animals for Johne's disease.

The constancy of the liver and spleen infections in untreated mice, their comparatively early occurrence, their ease and speed of assessment using the smear or histological methods, and the observed fact that the intensity of infection can be altered by, for example, the use of drugs, suggested that they may be of use as indicators in screening trials of prophylactic and curative agents.

CONCLUSIONS

Mice of the Swiss white strain were inoculated intraperitoneally with *M. johnei* and the course of infection studied up to a period of one year. The disease produced was compared with that in groups of mice treated with suramin or with cortisone.

The intensity of infection in the liver and spleens of the mice was studied using smear and histological techniques. Liver and spleen infections occurred in all the control mice but the intensity of infection varied between individual mice.

Intestinal infection with *M. johnei*, simulating classical Johne's disease, was detected in only a low proportion of mice.

Suramin administration enhanced the liver and spleen infections and appeared to enhance intestinal infections to a limited degree.

Cortisone administration reduced the liver and spleen infections, and had no observable effect on intestinal infection.

Under the conditions of the experiments, Swiss white mice appeared to be of limited value as experimental animals for the study of classical intestinal Johne's disease. However, the constancy of the liver and spleen infections, their comparatively early occurrence, and their ease of assessment by smear or histological methods, suggested that they may be of use as indicators in screening trials of therapeutic and prophylactic agents.

ACKNOWLEDGMENTS

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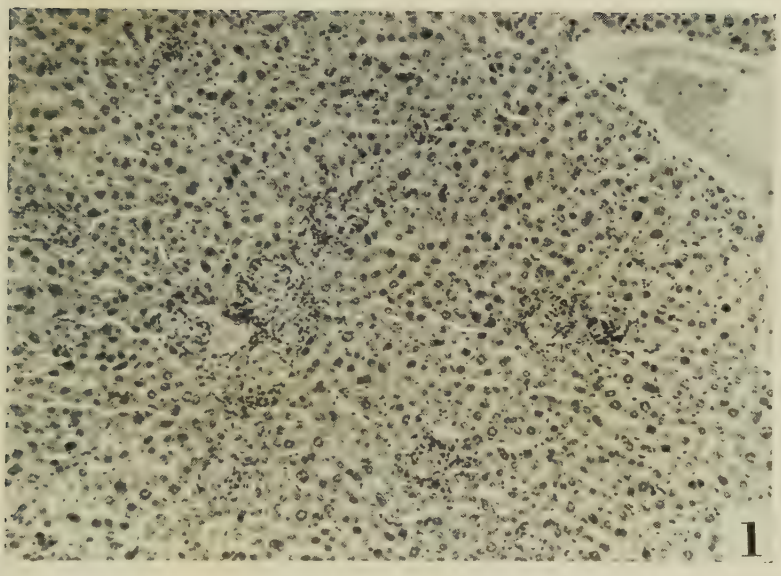

Fig. 1. Liver of mouse infected three months previously, showing "granuloma" type collections of macrophages. Stained by haematoxylin and eosin. × $\frac{2}{3}$ objective.

Fig. 2. As Fig. 1, but stained by the Ziehl-Neelsen method to show groups of bacilli.

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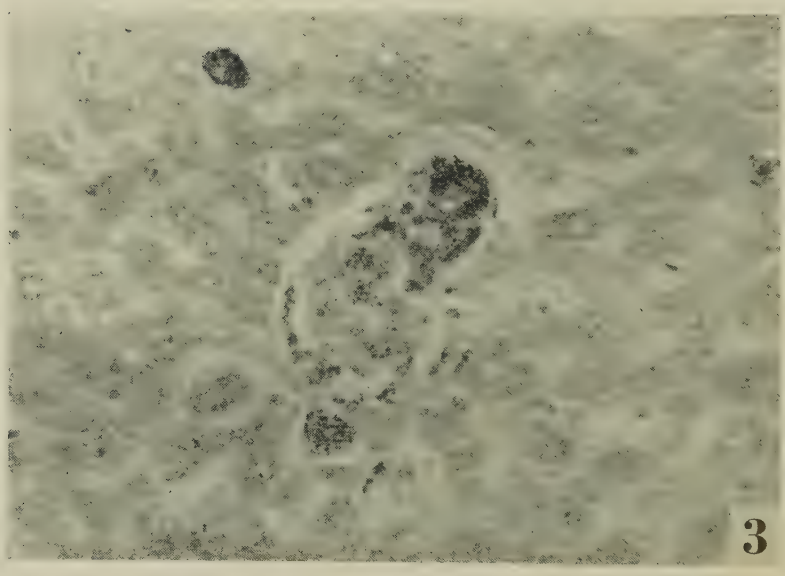


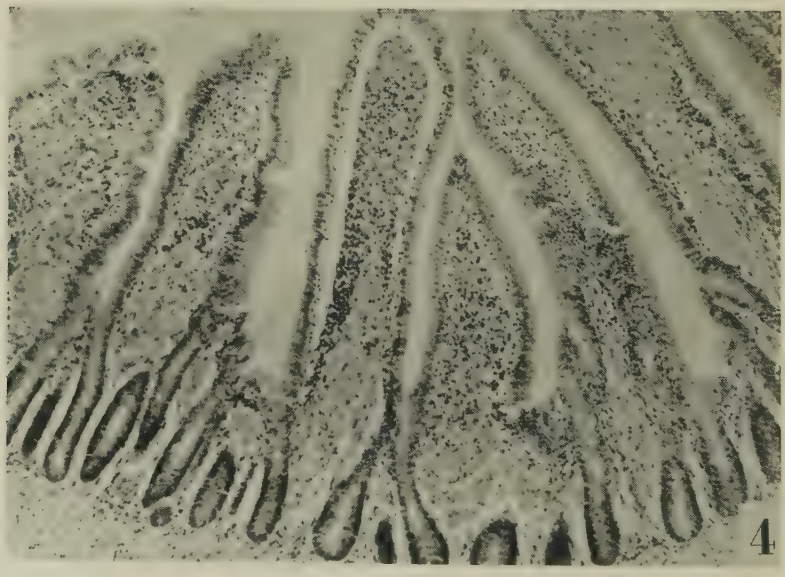
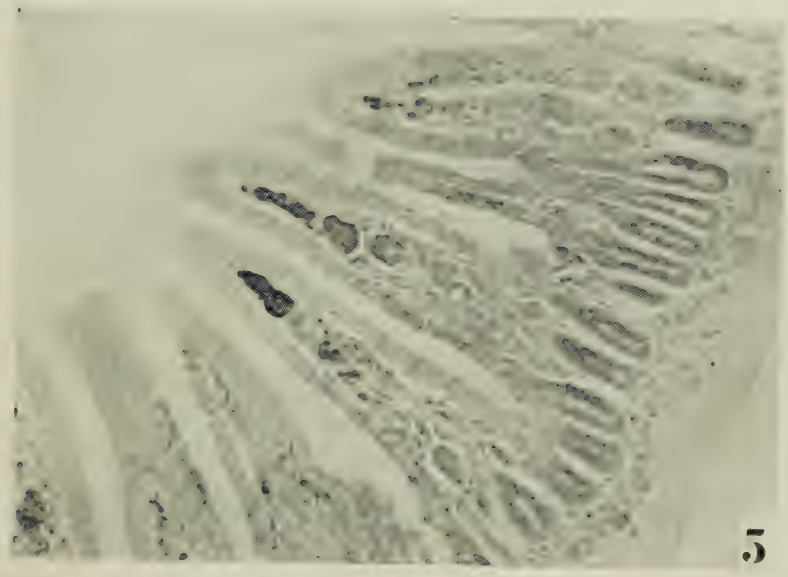


Fig. 3. As Fig. 2, but × $\frac{1}{6}$ objective, to show groups of bacilli and associated cells.

Fig. 4. Duodenum of mouse infected one year previously, showing masses of endothelial-type cells. The epithelial lining of the villi appears intact. Stained by haematoxylin and eosin. × $\frac{2}{3}$ objective.



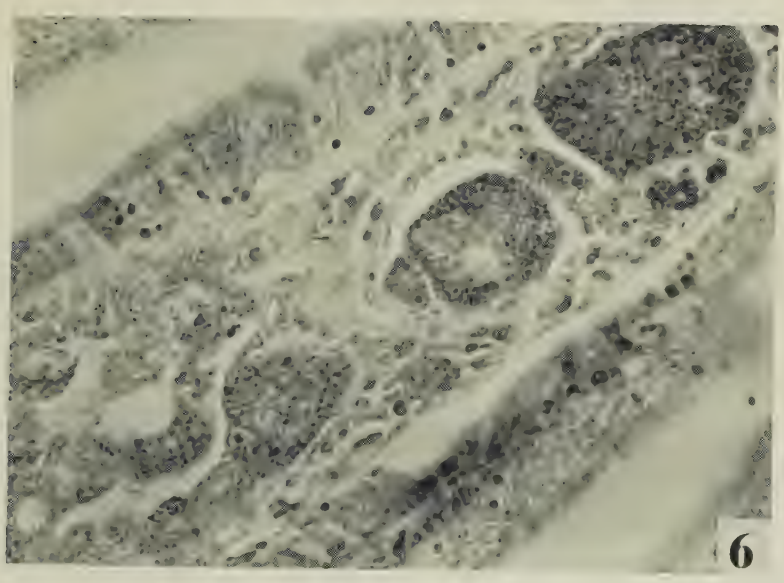


Fig. 5. As Fig. 4, but stained by Ziehl-Neelsen method to show groups of bacilli in the mucosa and submucosa.

Fig. 6. As Fig. 5, but × $\frac{1}{6}$ objective, to show groups of bacilli, and associated cells of one villus.

INFECTION OF LABORATORY ANIMALS WITH *MYCOBACTERIUM JOHNEI*

II. Infection in White Rats; Effect of Cortisone Treatment

By

R. L. Chandler

Agricultural Research Council Field Station, Compton, Berks.

INTRODUCTION

Reports on infection of the rat with *Mycobacterium johnei* by Twort (1913), Boquet (1925) and other workers have indicated that this animal is refractory. In view of the recent promise of success achieved by infecting other species of laboratory animals in early life and observing them for long periods, it was decided to examine the susceptibility of the rat in these respects, and also to observe any modification of the infection by cortisone. The methods adopted were similar to those used in the experiments on mice (Chandler, 1961a), and enabled some comparison of the infection between rats and mice to be made.

MATERIALS AND METHODS

Rats. These were of the white Wistar stock, which has been maintained as a closed colony at this Field Station for several years. The rats were infected when approximately four weeks old, and had been recently weaned.

Cultural methods. The methods (Chandler, 1961a) were qualitative and intended to give information regarding the presence of viable bacilli. All cultures were observed periodically for three months before being regarded as negative.

Strain of M. johnei. This strain had originally been isolated from a case of Johne's disease in a cow (Chandler, 1961a).

Inoculum. This was prepared by harvesting 6 to 7 week old cultures; a weighed quantity of moist bacilli was suspended in saline to constitute the required dose, which was inoculated intraperitoneally in a total volume of 0·5 ml. Inoculation was made between the second and third dose of cortisone.

Smear preparations. Impression smears of liver and spleen were stained by the Ziehl-Neelsen method and examined using a 1/12″ oil-immersion lens and x6 eyepiece. The extent of infection was estimated as the average number of bacilli seen per microscope field.

Histological preparations. Tissues were fixed in formalin and stained by the Ziehl-Neelsen method in all cases, and in some cases also by haematoxylin and eosin. An estimate of the liver and spleen infections was made by counting the numbers of bacilli seen per microscope field, using a 1/6 lens and x6 eyepiece, and the results recorded as the average number of groups of bacilli/number of fields. Sections of intestines were taken from the ileum and colon, near the ileo-caecal junction.

Dosage of Cortisone. Cortisone acetate*, diluted in sterile saline to give the required dose in 0·1 ml., was administered subcutaneously. A preliminary trial showed that a suitable dose regimen for cortisone was 4 mg./100 g. body-weight, each Monday, Wednesday and Friday for two weeks. This dosage temporarily reduced the rate of increase in weight of the uninfected rats and caused a reduction in their leucocyte cell counts, but did not cause sickness or death.

Plan of experiment. Group A consisted of forty-eight control rats; Group B consisted of forty-eight rats treated with cortisone. In each group, twenty-four rats were inoculated with 2 mg. *M. johnei* and twenty-four with 0·02 mg. *M. johnei.* Three months and six months after inoculation, six rats in each category were killed for examination, and the remaining rats were killed one year after inoculation.

RESULTS

Two weeks after commencement of the experiment thirteen rats in the cortisone treated Group B died. Pneumonia was a constant feature, nephritis and hepatitis also occurring in some cases; Pasteurella organisms were cultured from the livers and spleens and it is considered that the deaths were due to bacterial infection. It is likely that the cortisone administration reduced the resistance of the rats to this incidental infection. The results of the sampling and final examination are summarised in Table 1.

Throughout the experiment no macroscopic lesions that could be ascribed to *M. johnei* infection were seen. No bacilli or abnormalities were seen in the histological preparations of the intestines in any of the rats.

The three month sampling showed that in group A, not treated with cortisone, five of six rats given the higher dose of *M. johnei* had bacilli in small numbers (1 group per twenty or thirty fields) in the spleen preparations, and one of these rats showed bacilli in small numbers in a liver preparation, but all six rats were shown by the cultural method to harbour viable bacilli in both liver and spleen. Only the cultural method revealed infection in the six rats given the lower dose of *M. johnei.* In group B, treated with cortisone, bacilli in small numbers were seen only in two of the spleens of rats given the higher *M. johnei* dose; the cultural method revealed infection in all of the rats given the higher infective dose, and in four of the rats given the lower dose. The bacilli, when present, were usually associated with only minor tissue changes consisting of small groups of endothelioid cells.

The six month sampling similarly showed only low grade infections of the liver and spleen in the rats given the higher dose of *M. johnei.* The cultural method showed that all of the rats on the higher dose, but only half of those on the lower dose, harboured the organism.

The one year final examinations similarly showed only low grade

* Supplied by Glaxo Laboratories Limited.

TABLE I

INFECTION OF TWO GROUPS OF RATS, AT 3, 6, AND 12 MONTH INTERVALS

			Group A		*Group B*	
			Not drug treated		*Treated with Cortisone*	
			2 mg M. johnei	*0·2 mg M. johnei*	*2 mg M. johnei*	*0·2 mg M. johnei*
3 month sample	Liver	Smear	1+, 5−	6−	6−	6−
		Histology	6−	6−	6−	6−
		Culture	6+	6+	6+	4+, 2−
	Spleen	Smear	1+, 5−	6−	1+, 5−	6÷
		Histology	5+, 1−	6−	2+, 4−	6÷
		Culture	6+	6+	6+	4+, 2−
	Intestines	Histology	6−	6−	6−	6−
6 month sample	Liver	Smear	6−	6−	6−	6−
		Histology	6+	6−	6+	6−
		Culture	6+	6+	6+	5+, 1−
	Spleen	Smear	6−	6−	1+, 5−	6−
		Histology	2+, 4−	6−	1+, 5−	6−
		Culture	6+	3+, 3−	6+	3+, 3−
	Intestines	Histology	6−	6−	6−	6−
12 month sample	Liver	Smear	1+, 12−	10−	5−	4−
		Histology	3+, 10−	10−	5−	4−
		Culture	9+, 4−	5+, 5−	5+	4−
	Spleen	Smear	1+, 12−	10−	1+, 4−	4−
		Histology	2+, 11−	10−	1+, 4−	4−
		Culture	4+, 9−	1+, 9−	2+, 3−	4−
	Intestines	Histology	13−	10−	5−	4−

\+ = *M. johnei* detected
− = No *M. johnei* detected

infections in rats given the higher dose. In rats given the lower dose no bacilli were seen in preparations from the livers and spleens. No bacilli were detected, even on cultural examination, in the livers and spleens of the rats given the lower dose of *M. johnei* and treated with cortisone.

It was evident throughout the experiment that, even with the higher dose of *M. johnei*, the level of infection in the livers and spleens of the rats was very low. There was no evidence that cortisone treatment enhanced the infection, but, rather that the drug reduced the intensity of infection to a limited extent. Throughout the experiment the cortisone treated rats were heavier than the untreated rats; the dose of *M. johnei* given did not appear to influence the weight of the rats, whether cortisone treated or not.

DISCUSSION

The white rats used in this experiment showed no promise that they would be of use as laboratory animals for Johne's disease. No intestinal infections were detected, and the liver and spleen infections were slight. Infection shortly after weaning did not result in the appearance of typical signs of Johne's disease, and it seems unlikely that more significant lesions would have been detected had the period of observation been greater than the one year employed.

Viable bacilli were detected in the livers and spleens of the rats examined, especially three months and six months after infection, but one year after infection there was evidence suggesting that in some cases resolution of the infection had taken place. The cortisone treatment employed did not enhance the infection and may have reduced it.

The experiments using mice, described previously (Chandler, 1961a) when compared with the present experiments, clearly indicate that mice are of greater promise than rats as experimental animals for Johne's disease investigations.

CONCLUSIONS

Recently weaned white Wistar rats were infected with *M. johnei* and observed for a period of one year; one group of the rats was treated with cortisone at the commencement of the experiment.

No intestinal infections were detected. Infection of the liver and spleen occurred, but was not marked. Viable bacilli could be recovered from some of the rats one year after infection, but there was evidence of resolution of the infection at this time.

The cortisone treatment employed did not enhance the infection and may have reduced it.

It was concluded that rats are unlikely to be of use as laboratory animals for Johne's disease.

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INFECTION OF LABORATORY ANIMALS WITH *MYCOBACTERIUM JOHNEI*

III. Liver and Spleen Infections in Vaccinated and Unvaccinated Swiss White Mice

By

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INTRODUCTION

This investigation was designed to observe if the liver and spleen infections, in Swiss white mice infected with *Mycobacterium johnei*, can serve as indicators of the effect of prophylactic vaccines, as previously suggested (Chandler, 1961a). The criteria of intensity of infection were based on examinations of impression smears and histological sections; quantitative cultural examinations were avoided since the latter are lengthy due to slow growth of the organism and would limit the value of a screening test for vaccines.

MATERIALS AND METHODS

Mice. The mice were of the Swiss white strain and were vaccinated, newly weaned, when three or four weeks old.

M. johnei. The strain of *M. johnei* was one originally isolated from a case of Johne's disease in a cow; this strain was previously used to infect mice, from which it was re-isolated and subcultured. The dose of moist bacilli suspended in saline was administered in a volume of 0·25 ml. by the intraperitoneal route. In the two experiments to be described the inocula were prepared from different sub-inoculations of the strain.

Examinations. Liver and spleen impression smears, histological preparations and cultures were made and examined as already described (Chandler, 1961). Up to 100 fields were examined for each smear and at least 50 fields for each histological preparation, before estimating the intensity of infection.

*Weybridge vaccine.** This vaccine consisted of viable *M. johnei* suspended in equal parts of olive oil and liquid paraffin to a concentration of 5 mg. per 1·5 ml.; to this was added 10 mg. per 1·5 ml. of pumice powder as an irritant. The dose used in mice was 0·4 ml., inoculated subcutaneously.

Killed vaccine. This vaccine consisted of dried, heat-killed *M. johnei* suspended in liquid paraffin to a concentration of 10 mg. per ml. The dose used in mice was 0·2 ml., inoculated subcutaneously.

General effect of the vaccines. In a preliminary trial, groups of 10 to 20 mice each were inoculated with the selected vaccine, with liquid paraffin alone or left uninoculated as controls, and observed for 3 to 4 months. No loss in weight was noted in the vaccinated mice. The local reactions at the site of inoculation were in all cases benign, consisting of circumscribed lumps which did not ulcerate or appear painful. Impression smears of the livers and spleens were examined at the end of the trial; no bacilli were seen.

* Supplied by Weybridge Central Veterinary Laboratory by courtesy of Mr. H. N. Spears.

RESULTS

Mice Examined 2 Months and 4 Months after Challenge with 8 mg. and 2 mg.

Three groups each of 20 mice, were used; one group was inoculated with killed vaccine, one with the liquid paraffin adjuvant only and one was left as control. All three groups were inoculated with *M. johnei* three weeks later; two doses were used in each group, one of 8 mg. and one of 2 mg. Half the surviving mice were killed and examined two months, and the remaining mice four months after infection.

Early in the course of the experiment, five mice distributed in the various groups, died of causes which did not appear related to the interpretation of the experiment. The results of examinations of the surviving 55 mice are shown in Table 1. It will be seen that the 2 month examinations showed little or no evidence of protection due to the vaccine. The 4 month examinations showed no evidence of vaccine protection at the 8 mg. dose level; at the 2 mg. dose level the evidence suggested a degree of protection due to the vaccine (but not due to the adjuvant alone), the liver and spleen examinations of the vaccinated mice giving negative results or lower counts than the control mice.

Cultures of each liver and spleen were positive, indicating the presence of viable bacilli, except in the case of one mouse that had been vaccinated, inoculated with the 8 mg. dose and examined 4 months later.

Mice Examined 4 Months after Challenge with 2 mg. and 0·2 mg.

Three groups, each of 24 mice were used; one group was vaccinated with Weybridge vaccine, one with killed vaccine and one was left as control. All three groups were inoculated with *M. johnei* one month later; two doses were used, one of 2 mg. and one of 0.2 mg. All the surviving mice were killed and examined four months after infection.

In the course of the experiment 8 mice, distributed in the various groups, died of causes which did not appear relevant to the interpretation of the experiment. The results of examinations of the surviving 64 mice are shown in Table 2.

At the 0·2 mg. dose level, the majority of both smear and histological preparations were negative, indicating that this dose was insufficient for the purpose of the examination techniques, and no conclusions regarding differences between the groups could be drawn. At the 2 mg. dose level, all the liver smears either gave low counts, varying from 1 group to 8 groups of bacilli per 100 microscope fields, or were negative. Both vaccine groups had a greater number of negative examinations, suggesting vaccine protection. The spleen smears all gave low counts, the killed vaccine group having the lowest intensity of infection. The liver histological examinations showed that both vaccine groups had negative specimens, although some showed pleiomorphic Ziehl-Neelsen positive material suggestive of bacterial

TABLE 1

EXAMINATIONS MADE TWO MONTHS AND FOUR MONTHS AFTER CHALLENGE

Time after Challenge	*Challenge dose*	*Group*	*No. of mice*	*Liver smear*	*Spleen smear*	*Liver section*	*Spleen section*
Two months	8 mg	Control	5	4 + 1 ⊖	3 + 2 ⊖	5 +++	1 +++ 4 ++
		Vaccine	5	1 ++ 4 +	2 ++ 3 +	5 +++	2 +++ 3 ++
		Adjuvant	5	1 ++ 3 + 1 ⊖	5 ++	4 +++ 1 ++	4 ++ 1 +
	2 mg	Control	5	5 +	4 + 1 ⊖	3 +++ 2 ++	5 ++
		Vaccine	4	3 + 1 ⊖	3 + 1 ⊖	3 +++ 1 ++	2 ++ 2 +
		Adjuvant	5	5 +	4 + 1 ⊖	2 +++ 3 ++	4 ++ 1 +
Four months	8 mg	Control	4	1 ++ 3 +	4 +	1 +++ 3 ++	1 +++ 3 ++
		Vaccine	4	1 ++ 3 +	4 +	2 +++ 2 ++	1 +++ 3 ++
		Adjuvant	4	1 ++ 3 +	1 ++ 3 +	2 +++ 2 ++	1 +++ 1 ++ 1 + 1 ⊖
	2 mg	Control	5	1 ++ 4 +	1 ++ 4 +	2 +++ 3 ++	1 +++ 4 ++
		Vaccine	4	2 + 2 ⊖	2 + 2 ⊖	2 ++ 2 +	1 ++ 3 +
		Adjuvant	5	2 ++ 3 +	1 ++ 4 +	4 +++ 1 ++	2 +++ 2 ++ 1 +

The symbols representing intensity of infection in the smear examinations

++ = more than one group of bacilli/10 microscope fields
+ = less than one group of bacilli/10 microscope fields
⊖ = no bacilli seen

The symbols representing intensity of infection in the histological section examinations

+++ = more than one group of bacilli/3 microscope fields
++ = one group of bacilli/3 to 15 microscope fields
+ = less than one group of bacilli/15 microscope fields

TABLE 2

EXAMINATIONS MADE FOUR MONTHS AFTER CHALLENGE

Challenge dose	*Group*	*No. of mice*	*Liver smear*	*Spleen smear*	*Liver section*	*Spleen section*	*Spleen Wt. / Body Wt. × 100%*	
							Mean	*Range*
2 mg.	Control	10	7 + 3 ⊖	1 ++ 7 + 2 ⊖	7 ++ 3 +	7 ++ 2 + 1 ⊖	0·94	0·77 — 1·2
	Weybridge vaccine	9	4 + 5 ⊖	2 ++ 5 + 2 ⊖	1 ++ 3 + 4 D 1 ⊖	2 ++ 1 + 6 ⊖	0·98	0·42 — 1·6
	Killed vaccine	11	3 + 8 ⊖	6 + 5 ⊖	6 ++ 3 + 1 D 1 ⊖	2 ++ 4 + 5 ⊖	1·02	0·7 — 1·9
0·2 mg.	Control	12	1 + 11 ⊖	3 + 9 ⊖	2 + 10 ⊖	1 ++ 1 + 10 ⊖	0·78	0·6 — 1·4
	Weybridge vaccine	12	1 + 11 ⊖	4 + 8 ⊖	3 + 9 ⊖	2 ++ 2 + 8 ⊖	0·84	0·5 — 1·9
	Killed vaccine	10	2 + 8 ⊖	2 + 8 ⊖	2 + 8 ⊖	3 + 7 ⊖	0·82	0·52 — 1·4

For interpretation of symbols see Table 1.

débris. The spleen histological specimens showed that both vaccine groups had a high proportion of negative specimens.

The ratios $\frac{\text{spleen weight}}{\text{body weight}}$ were higher in the three groups given the 2 mg. dose than in the three groups given 0·2 mg. There was little difference between the vaccinated and control groups, the ratios being a little higher in the vaccinated groups.

DISCUSSION

The results of both experiments suggested that a degree of protection due to vaccines could be detected in the mice, when a challenge dose of 2 mg. *M. johnei* was used and the mice were examined four months later. However, a decisive and easily interpreted result was not obtained in either experiment; this may have been due to the individual differences in susceptibility to infection of Swiss white mice.

CONCLUSIONS

Groups of Swiss white mice were vaccinated and challenged with *M. johnei*. Estimations were made of the intensity of infection in their livers and spleens by examination of impression smears and by histological sections.

The results suggested that a degree of protection due to vaccines could be detected when a challenge dose of 2 mg. *M. johnei* was used, and the mice were examined four months later.

A decisive and easily interpreted result was not obtained and it is considered that Swiss white mice, with the methods used, could not be used as a standard screening test for the study of vaccines against Johne's disease.

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SOME FACTORS INFLUENCING THE PROPAGATION OF RINDERPEST VIRUS IN EMBRYONATED EGGS

By

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INTRODUCTION

The lapinised-avianised strain of rinderpest virus (LA virus) was developed by Nakamura and Miyamoto (1953) from the Nakamura III strain of lapinised rinderpest virus which was originally described by Nakamura, Wagatuma and Fukusho in 1938. As a result of its adaptation to the chick embryo the virus has reached a higher degree of attenuation for cattle than that of the lapinised virus and is particularly suitable, therefore, for the immunisation of highly susceptible cattle. In studies on the LA virus Nakamura, Kishi and Miyamoto (1954) found that inoculation of the eggs by the intravenous route resulted in the highest yield of virus and this method was adopted as a routine using 12- to 13-day old White Leghorn embryos.

Further studies have been carried out to investigate the influence of the age and breed of the embryos on the propagation of the virus.

MATERIALS AND METHODS

Virus. The LA strain of virus used was between generations A-E 592 and A-E 604. This was the result of from 669 to 681 chick embryo passages with 41 rabbit passages.

Eggs. For the study of the age factor 10-, 12- and 14-day old White Leghorn embryos were used. For the study of the breed factor 12-day old White Leghorn, Barred Plymouth Rock and Rhode Island Red embryos were compared. All virus titrations were carried out in 12-day old White Leghorn embryos.

Inculum. A 1:2000 suspension of infected chick embryo spleens in 6 per cent. glucose was used as the source of virus. All eggs were inoculated intravenously with 0·05 ml. of inoculum.

In the study of the age factor, groups of 10-, 12- and 14-day old White Leghorn embryos were inoculated with the 1:2000 suspension. The eggs were re-incubated for four days and then the surviving embryos were harvested. The head, wings and legs were removed and the embryos were taken up in 0·9 per cent buffered saline (pH 7·2) to make a 1:5 suspension. Part of this suspension was set aside for the preparation of complement-fixing antigen. The remainder was centrifuged for 15 minutes at 4,000 r.p.m. and the supernate used for the titration of the virus. Tenfold dilutions were prepared from the supernate, from 10^{-3} to 10^{-7}, and the virus was titrated in 12-day old White Leghorn eggs, 6 eggs per dilution. These eggs were re-incubated for four days and the surviving embryos harvested. The livers and spleens were removed from the embryos and used for the demonstration of complement-fixing antigen.

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Complement-fixation Tests

Antigens. The uncentrifuged embryo body suspensions were boiled for 30 minutes and stored overnight at 4°C. They were then centrifuged and the supernate used as antigen. The livers harvested from the virus titration were taken up in saline individually to make 1:10 suspensions. These were treated in the same way as the embryo suspensions. As the chick embryo spleens were too small to be tested individually, the spleens from each dilution group were pooled and prepared in the same manner as the livers, the only other difference being that they were made up in a 1:20 suspension in order to give a sufficient volume of antigen for testing.

Complement fixation. The technique used was the titration of residual complement in miniature system developed by Nakamura and the method and materials have been described elsewhere (Nakamura, 1958).

Breeds. For the study of the breed factor a similar procedure was used, substituting the eggs of White Leghorn, Barred Plymouth Rock and Rhode Island Red hens. The virus titrations were again carried out in White Leghorn eggs.

RESULTS

The results of the comparison of the age groups are given in Table 1. It will be seen that, with the exception of Experiment 3, the antigens prepared from the 10-day old embryo bodies were stronger than those of the 12-day and 14-day old groups. The differences in the three groups were also apparent from the results of the virus titration. These results are expressed in terms of 50 per cent egg infective doses (EID_{50}) which were calculated by the method of Reed and Muench (1938). The EID_{50} of the virus grown in the 10-day old embryos varied from half a log (Experiment 1) to almost two logs (Experiment 2) higher than that of the virus grown in the 12-day old embryos. In all three experiments the 14-day old embryos were quite unsatisfactory. When the pooled spleens from each dilution group of the titrations were tested the differences were not so apparent, but, since one infected spleen in a group would be sufficient to give a positive reaction in the C.F. test, these figures do not give an accurate estimate of the amount of virus present at a given dilution, but only indicate the presence or absence of virus at that dilution.

In Table 2 the results of the comparison of the eggs from three breeds are given. It can be seen from the demonstrations of antigen in the embryo bodies that there was apparently little difference in the three groups. However, when the virus content was titrated the virus grown in the White Leghorn embryos had a titre of more than 10^{-4} in two of the three experiments while that from the Barred Plymouth Rock embryos reached a titre of 10^{-4} in only one experiment and the titre of the virus grown in the Rhode Island Red embryos did not exceed $10^{-3.5}$. Also, the virus was present at a dilution of 10^{-5} in the material from the White Leghorn eggs in two of the three experiments, but was a log less in the other two breeds.

TABLE 1

INFLUENCE OF THE AGE FACTOR OF THE EGGS ON THE PROPAGATION OF VIRUS

Age of eggs Inoculated Days	*Titre of CF antigen in embryo body expressed as two-fold dilution*			*Virus titre in embryo body*					
				Liver EID_{50}			*Spleen*		
	Experiment			*Experiment*			*Experiment*		
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
10	1:160	1:160	1:320	$10^{-4.4}$	$10^{-4.3}$	$10^{-4.5}$	10^{-5}	10^{-5}	10^{-5}
12	1:40	1:10	1:320	$10^{-3.8}$	$\leq 10^{-2.5}$	$10^{-4.5}$	10^{-5}	10^{-4}	10^{-5}
14	$\leq$1:5	$\leq$1:5	80	$\leq 10^{-2.5}$	$\leq 10^{-2.5}$	$\leq 10^{-2.5}$	10^{-3}	10^{-3}	10^{-3}

TABLE 2

INFLUENCE OF BREED FACTOR ON THE PROPAGATION OF VIRUS

Breed of eggs inoculated 12-day old	*Titre of CF antigen in embryo body expressed as two-fold dilution*			*Virus titre in embryo body*					
				Liver EID_{50}			*Spleen*		
	Experiment			*Experiment*			*Experiment*		
	1	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
White Leghorn	1:160	1:80	1:80	$10^{-3.6}$	$10^{-4.4}$	$10^{-4.5}$	10^{-5}	10^{-4}	10^{-5}
Barred Plymouth Rock	1:160	1:40	1:80	$10^{-2.7}$	$10^{-3.3}$	$10^{-4.2}$	10^{-4}	10^{-4}	10^{-4}
Rhode Island Red	1:160	1:20	1:80	$10^{-3.5}$	$10^{-3.5}$	$10^{-3.4}$	10^{-4}	10^{-4}	10^{-4}

DISCUSSION

The difference in the three age groups of the White Leghorn embryos was assessed in three ways; firstly, by the demonstration of antigen in the embryo bodies, secondly, by the calculation of the EID_{50} and thirdly, by the demonstration of antigen in the pooled spleens. The antigens prepared from the embryo bodies were tested in two-fold dilutions. The results showed that there was a marked difference in the strengths of the antigens. According to Nakamura (1958) the development of complement-fixing antigen is roughly in parallel with the propagation of the virus. It is assumed, therefore, that the differences observed could be the result of different degrees of virus propagation. The most satisfactory method of showing this was to measure the quantities of virus in terms of EID_{50}. The results showed the superiority of the 10-day old embryos over the 12-day and 14-day embryos. It has already been stated that the results obtained from the tests carried out on the pooled spleens do not give an accurate estimate of the amount of virus present. However, it has been shown (Nakamura, 1958) that for the detection of a weak infection, the spleen is the organ of choice and the figures given represent the highest dilution of inoculum which contained an infective dose of virus.

In practice, the intravenous inoculation of the 10-day old eggs was no more more difficult than the inoculation of the older eggs, nor was there any difference in mortality due to the inoculation.

Three preliminary experiments were carried out using 12-day old eggs to assess the possible influence of the breed of embryo on the propagation of the virus. The results obtained suggest that the White Leghorn embryos may be superior to the Barred Plymouth Rock and Rhode Island Red breeds. However, while these experiments were in progress the results of the work on the age factor showed that with White Leghorn embryos at least, 10 days was the optimum age for virus propagation; hence, before an accurate comparison of the three breeds can be made it will be necessary to establish the optimum age for the Barred Plymouth Rock and Rhode Island Red embryos.

CONCLUSIONS

For the propagation of the LA strain of rinderpest virus in eggs, the best results are obtained using 10-day old embryos. The use of eggs over 12 days old is not recommended.

The breed of bird providing the embryos may also have an influence on the growth of the virus, but further work is necessary before the extent of this breed factor can be assessed.

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FURTHER IMMUNOLOGICAL STUDIES IN EXPERIMENTAL STAPHYLOCOCCAL MASTITIS*

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INTRODUCTION

In a previous publication (Derbyshire, 1960) it was shown that vaccination of goats and cows with an adjuvant cell-toxoid prepared from *Staph. aureus* strain 201 conferred a high level of protection against challenge by mammary inoculation of massive numbers of staphylococci of the homologous strain. The present paper describes immunological studies in goats involving certain other strains of staphylococci which were selected for study because they differed from strain 201 in one or more characteristics.

MATERIALS AND METHODS

Characteristics of Strains of Staphylococci

The main characteristics of the strains used in these studies are given in Table 1; these were determined by the tests detailed below. The phage-pattern of each strain was tested.

Rabbit and sheep cell lysis. Strains were grown in 0·3 per cent Bacto-tryptose agar for four days in an atmosphere of 25 per cent carbon dioxide in air. The agar was removed by coarse paper and the toxins passed through Seitz EKS pads under 250 mm. Hg negative pressure. The filtrates were then titrated for haemolytic activity against equal volumes of 2 per cent suspensions of thrice washed rabbit and sheep red blood cells. The rabbit cell tests were incubated at 37°C for one hour, and the sheep cells for two hours at 37°C followed by 18 hours at 4°C. The figures in Table 1 are the dilutions of filtrates which caused 50 per cent lysis of the red cells.

Delta lysis. A strain was designated as a delta lysin producer if it produced on a rabbit blood agar plate haemolysis which was not inhibited by a filter paper strip soaked in alpha antitoxin 750 units per ml. placed at right angles to the streak of culture.

Mouse lethal toxin. Filtrates prepared as for the haemolytic tests were injected intravenously into 20 g. female White Swiss mice. The minimal lethal doses of the filtrates are recorded in Table 1.

Free coagulase. Broth culture supernatants were prepared and assayed for free coagulase activity as described by Duthie and Lorenz (1952). The figures shown in Table 1 indicate the dilutions of supernatants which produced minimal clotting.

Bound coagulase. The production of bound coagulase was demonstrated by the slide method of Cadness-Graves, Williams, Harper and Miles (1943).

*The work described in this paper forms part of a thesis approved by the University of London for the award of the Ph.D. Degree.

Hyaluronidase. The hyaluronidase activity of a Seitz EKS filtrate of an 18 hour culture in Todd-Hewitt broth was assayed as described by Howell (1954) and is expressed in Table 1 as the greatest dilution of filtrate which caused spreading of the substrate.

Fibrinolysis. Fibrinolysis was demonstrated on heated 12 per cent rabbit plasma agar plates, after the method of Christie and Wilson (1941). Clearing of the medium is designated '+' in Table 1.

Protease. Agar plates containing 5 per cent goat blood which had been laked by repeated freezing and thawing were used to demonstrate the destruction of haemoglobin by staphylococci, which were streaked on the surface of the plates. In Table 1 a wide zone of clearing is indicated as '++', a narrow zone as '+' and a trace of clearing as '±'.

Gelatinase. Strains were inoculated into nutrient gelatin, which was incubated at 37°C for five days and held at 4°C for 30 minutes daily, when gelatinase activity was indicated by failure of the medium to solidify. This is shown as '+' in Table 1.

Lipase. Lipase activity was demonstrated by the production of clearing on nutrient agar plates containing 0·2 per cent tributyrin. Zones of clearing are designated in Table 1 as '++', '+' and '±', as for protease production.

LD_{50} for mice. Dilutions of 18 hour broth cultures of each strain were prepared in sterile broth, and each dilution inoculated intraperitoneally into ten 20 g. female White Swiss Mice, and the latter observed for deaths for ten days. In Table 1 the approximate LD_{50} is expressed as numbers of living cocci.

Preparation of Vaccines

Adjuvant cell-toxoid was prepared from the appropriate strain of staphylococcus and administered to goats as described previously (Derbyshire, 1960).

Serum Antibody Tests

Samples of blood serum were heated in a water-bath at 56°C for 30 mins. before testing.

Agglutination, gel diffusion precipitin, antihaemolysin, free coagulase inhibitor and mouse protection tests. These were performed as described by Derbyshire (1960).

Antibody to bound coagulase. This was measured by the method of Duthie (1954).

Hyaluronidase inhibitor. The test used was that of Howell (1954).

Inhibitors to fibrinolysin, lipase and protease. Simple qualitative plate tests were employed to test for inhibition of these enzymes by samples of serum, based on the techniques mentioned by Elek (1959).

Phagocytic power of circulating neutrophils. This test was conducted on whole blood, and not inactivated serum. The method used was a modification of the opsonic tests for *Brucella abortus* described by Tovar (1944) and Larsen (1950). Blood (0·9 ml.) was withdrawn from the jugular vein into 0·1 ml. sterile 4 per cent sodium citrate. To this was added 1 ml. of a suspension in saline matching Brown's tube 8 of formalin-killed washed staphylococci from an 18-hour broth culture. The tubes were then incu-

bated in a water bath at 37°C for 30 minutes, with hand agitation every five minutes. A film was prepared from each tube and stained with Giemsa; the number of cocci engulfed by each of 25 neutrophils in each film was counted and recorded as an index as follows: none = 0, 1 to 10 = 1, 11 to 20 = 2, 21 to 30 = 3, over 30 = 4. Thus for any test the phagocytic index could range from 0 to 100.

Experimental Production of Mastitis

The methods used for producing and characterising experimental mastitis in the goats were those which have been described in earlier papers (Derbyshire, 1958a, b; 1960).

RESULTS

Challenge of Immunity Produced by Strain 201 Adjuvant Cell Toxoid by Heterologous Strains (Exp. 1)

This experiment was designed to test the protective power of adjuvant cell-toxoid prepared from strain 201 against experimental mastitis produced by strains 106, BB, N90 and S20T. For each of these heterologous strains, two goats were vaccinated with strain 201 adjuvant cell-toxoid and one goat left as a control. In addition, one vaccinated and one control goat were challenged by strain 201 to check the potency of the vaccine. Antibody tests were made on serum samples taken from each goat at weekly intervals from one week before the first injection of vaccine up to the date of challenge. Sera were tested for alpha and beta antihaemolysin, and for agglutination, precipitation, coagulase inhibition and mouse protection against strain 201, and also against the appropriate challenge strain, except that a coagulase inhibition test had to be omitted for strain 106 because this strain did not yield a sufficiently potent coagulase for use. The challenge inoculum consisted of 10 ml. whole broth culture of the appropriate strain, containing approximately 10^9 living cocci, into the left teat canal of each goat. The experiment was terminated five weeks after challenge inoculation when the udders of surviving goats were submitted to histological examination.

Antibody production. The antibody levels at the time of challenge inoculation are shown in Table 2, and the salient features of the response are noted below.

Agglutinins. In the vaccinated goats, slight increases in agglutinin titres to strain 201 were observed, but increases in titre to the heterologous strains did not occur.

Precipitins. The sera from the vaccinated goats gave positive gel diffusion precipitin reactions with an acid extract of strain 201, and these also gave positive reactions with all the heterologous challenge strains which were used.

Antihaemolysins. High levels of alpha and beta antihaemolysin developed in the vaccinated goats; the non-vaccinated goats all had low antihaemolysin levels.

TABLE 1

CHARACTERISTICS OF STRAINS OF STAPHYLOCOCCI

	Strain No.				
	201	*106*	*BB*	*N90*	*S20T*
Source:					
Species	Cow	Cow	Cow	Cow	Sheep
Type of infection	Chronic mastitis	Acute mastitis	Chronic mastitis	Milk carrier	Acute mastitis
Phage pattern	42D	80	78	78	47A/78
Rabbit cell lysis	5120	1280	640	640	1280
Sheep cell lysis	1280	10240	5120	1280	10240
Delta lysis	+	+	+	+	+
Mouse lethal toxin (M.L.D.)	0·0031 ml.	0·05 ml.	0·1 ml.	0·5 ml.	0·025 ml.
Free coagulase	32	4	128	32	32
Bound coagulase	—	+	+	+	+
Hyaluronidase	512	8192	16	4	8
Fibrinolysis	+	—	—	—	+
Protease	++	+	±	+	+
Gelatinase	+	+	+	+	+
Lipase	+	—	±	±	+
LD_{50} for mice	18×10^6	25×10^6	21×10^6	21×10^6	15×10^6

TABLE 2

ANTIBODY LEVELS AND REACTION TO CHALLENGE BY HETEROLOGOUS STRAINS OF GOATS

Goats vaccinated with strain 201 adjuvant cell-toxoid

Challenge strain	*Goat No.*	*Serum antibody levels at time of challenge*										*Reaction to challenge*
		Agglutinins		*Precipitins*		*Antihaemolysins*		*Coag. inhibitor*		*Mouse protection*		
		Against strain 201	*Against challenge strain*	*Against strain 201*	*Against challenge strain*	*Alpha (units)*	*Beta (units)*	*Against strain 201*	*Against challenge strain*	*Against strain 201*	*Against challenge strain*	
201	111 V	160		+		64	256	0		4		Mild mastitis
	112 C	40		—		2	1	0		0		Gangrenous mastitis
106	113 V	160	20	+	+	32	32	0		4	2	Mild mastitis
	114 V	80	20	+	+	128	64	0		8	2	,, ,,
	115 C	80	20	—	—	1	1	0		0	0	Severe mastitis
BB	116 V	160	20	+	+	32	64	0	0	4	0	Severe mastitis
	117 V	320	40	+	+	256	64	0	0	8	0	,, ,,
	118 C	40	20	—	—	8	4	0	0	0	0	Gangrenous mastitis
N90	119 V	160	20	+	+	32	32	0	0	8	0	Severe mastitis
	120 V	80	20	+	+	32	64	0	0	4	0	,, ,,
	121 C	160	40	—	—	2	8	0	0	0	0	Gangrenous mastitis
S20T	122 V	80	20	+	+	128	64	0	0	2	0	Mild mastitis
	123 V	320	160	+	+	256	128	0	0	4	0	Severe mastitis
	124 C	80	40	—	—	1	4	0	0	0	0	,, ,,

The agglutinin titres are reciprocals of serum dilutions; the mouse protection titres are the reciprocals of the highest dilution of serum which protected mice.

V = goats vaccinated with strain 201 adjuvant cell-toxoid
C = non-vaccinated goats.

Coagulase inhibitor. The sera from all goats failed to inhibit the clotting action of coagulase of the strains of staphylococci which were tested.

Mouse protection test. Sera from the vaccinated goats conferred passive protection to mice against challenge by strain 201 and also protected mice against challenge by strain 106. No significant protection was evident, however, when mice were challenged by strains BB, N90 or S20T.

Reaction to mammary challenge. The results of the mammary challenge infections are also summarised in Table 2. The high level of protection against the homologous strain reported previously for this vaccine was confirmed in Goat 111, which showed a mild reaction only. A similarly high level of protection was apparent also against strain 106, which produced severe mastitis with total cessation of milk secretion in the control goat (115) but only mild transient reactions in the vaccinates. No such high level of protection was apparent against strains BB, N90 and S20T and each produced severe, purulent mastitis in one or both of the vaccinated animals. It is to be noticed that none of these strains produced gangrenous mastitis in a vaccinated goat, and it could be inferred that the modification in the pathogenesis of the experimental disease from gangrene to severe purulent mastitis was an expression of some degree of immunity. It was clear, however, that immunity against strains BB, N90 and S20T was of a lower grade than against strains 201 and 106. To determine whether this might be due to the toxins of strains resembling BB being of different antigenic type to those of strain 201, in the next experiment goats were vaccinated with adjuvant cell-toxoid prepared from strain BB and challenged with the homologous strain.

Attempt to Immunise Goats against Strain BB by Vaccination with Strain BB Adjuvant Cell-toxoid (Exp. II)

Four goats were vaccinated with strain BB adjuvant cell-toxoid and two goats included as non-vaccinated controls. The challenge inoculum consisted of 10 ml. whole broth culture of strain BB, containing approximately 10^9 living cocci, injected into the left teat canal. Histological examination of the udders was made one week after challenge. Serum samples were obtained from each goat at weekly intervals from one week before vaccination to the time of challenge. The samples were tested for alpha and beta antihaemolysins, gel diffusion precipitins against strain BB, for inhibitor to BB coagulase and for passive protection in mice against strains BB and 201.

Antibody production. The antibody levels in both groups of goats at the time of challenge are given in Table 3. The non-vaccinated goats had consistently low antihaemolysin levels, and gave negative reactions to the precipitin, coagulase inhibitor and mouse-protection tests. In the vaccinated goats, the production of beta antihaemolysin

TABLE 3

ANTIBODY LEVELS AND REACTION TO CHALLENGE WITH STRAIN BB

Goats vaccinated with strain BB adjuvant cell-toxoid

Goat No.	*Treatment*	*Antibody levels at time of challenge*						*Reaction to challenge*
		Precipitins	*Alpha antihaemolysin (units)*	*Beta antihaemolysin (units)*	*Coagulase inhibitor*	*Mouse protection*		
						Against BB	*Against 201*	
131	Untreated	—	1	1	0	0	0	Gangrenous mastitis
132	,,	—	0·5	1	0	0	0	,, ,,
133	Vaccinated	+	32	256	0	0	2	Severe mastitis
134	,,	+	64	256	0	0	2	,, ,,
135	,,	+	16	64	0	0	1	,, ,,
136	,,	+	64	256	0	0	4	,, ,,

The mouse protection titres are reciprocals of the highest dilution of serum which protected mice.

was similar to that which occurred in response to vaccination with strain 201 adjuvant cell-toxoid, but the alpha response was somewhat lower. Precipitins to strain BB were produced in the vaccinated goats, but no coagulase inhibitor was detected. An important observation was that serum from these goats did not confer passive protection on mice challenged by strain BB, but did confer significant protection on mice against challenge by strain 201.

Reaction to mammary challenge. The reactions of these goats to challenge inoculation are summarised in Table 3. Gangrenous mastitis rapidly developed in both the non-vaccinated controls. The reaction of the vaccinated goats resembled that of the 201-vaccinated goats which were challenged by strain BB in the previous experiment. They developed severe purulent mastitis, characterised by a persistent systemic reaction and total cessation of milk secretion from the inoculated sides of the udders. On histological examination of the udders, necrosis, abscess formation, ductal ingrowths and epithelial hyperplasia which have been described as typical of severe staphylococcal mastitis (Derbyshire, 1958b) were observed. Thus strain BB was able to produce severe mastitis in goats with a high level of immunity to the haemolysins of BB, and whose sera were capable of protecting mice against staphylococci of strain 201. In an attempt to demonstrate antigenic differences which might account for this behaviour, the next experiment was planned to compare the production of a variety of antibodies in goats experimentally infected with strain 201 with that in goats infected with strain BB.

Development of Antibodies in Experimental Strain 201 and Strain BB Mastitis (Exp. III)

The development of antibodies in goats with experimental mastitis produced by strain 201 was compared with that in goats

TABLE 4

GOATS USED IN MEASUREMENTS OF THE DEVELOPMENT OF ANTIBODIES IN EXPERIMENTAL MASTITIS

Goat No.	*Strain No.*	*No. of cocci in inoculum*	*Type of mastitis produced*
51	201	10^7	Severe
52	,,	,,	Gangrenous
53	,,	,,	Mild
54	,,	10^5	Mild
55	,,	,,	Severe
56	,,	,,	Severe
141	BB	10^7	Gangrenous
142	,,	,,	Severe
143	,,	,,	Gangrenous
144	,,	10^5	Mild
145	,,	,,	Severe
146	,,	,,	Severe

experimentally infected with strain BB, in an attempt to demonstrate antigenic differences between the two strains which might be important from the immunological point of view. The inoculation details on which these observations were made are given in Table 4. Serum samples were taken at weekly intervals from one week before mammary inoculation to six weeks after inoculation. All the antibody tests under "Materials and Methods" were carried out except for antibody to strain 201 bound coagulase and inhibitor to strain BB fibrinolysin, since these substances are not produced by these strains. Serum from the BB-infected goats was tested for mouse-protection against strain 201, in addition to strain BB.

Strain 201. Post-inoculation measurements of antibody levels were not made on Goat 52 because it was slaughtered when it developed gangrenous mastitis shortly after infection. In the goats which developed mild mastitis (53 and 54) the only change detected in the serum antibody levels following mammary inoculation was a transient increase of 4 to 8 units in serum alpha and beta antihaemolysin which was detected one week after inoculation. The development of antibodies in the goats in which severe mastitis occurred (51, 55 and 56) is described below, and the main findings are depicted graphically for Goat 55 in Fig. 1.

Agglutinins. All goats had an agglutinin titre of 1:80 or 1:160 before mammary inoculation. The titre increased up to 1:640 as mastitis developed.

Fig. 1

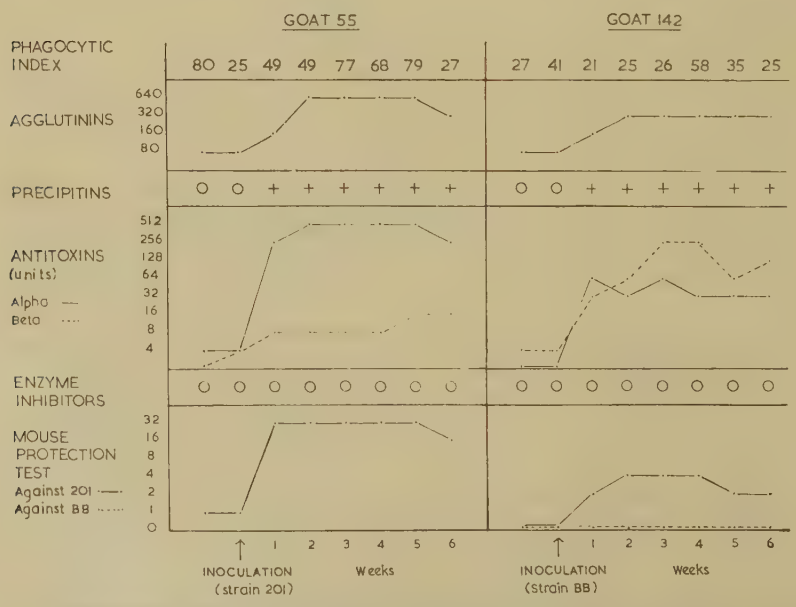

Fig. 1. The development of antibodies in experimental strain 201 mastitis (Goat 55) and experimental strain BB mastitis (Goat 142).

Precipitins. Sera were negative to the gel diffusion test before inoculation, but gave positive reactions when the disease developed. The serum of Goat 55, which showed a very severe mastitic reaction, remained positive throughout the period of the experiment, but in Goat 51 a positive reaction was given at only two samplings after inoculation, and in this goat the mastitis was less severe, with a smaller reduction in milk yield from the inoculated gland.

Antihaemolysins. High levels of alpha antihaemolysin rapidly developed after inoculation and highest levels were attained when the severity of the mastitis was greatest. The beta antihaemolysin levels of the sera followed a similar pattern but the increase in titre was always quantitatively much less.

Coagulase and hyaluronidase inhibitors. Neither of these antibodies was detected in any sample of serum.

Inhibitors to fibrinolysin, lipase and protease. The plate method used did not show inhibition of any of these enzymes.

Mouse-protecting antibody. Before inoculation, samples of serum protected mice only when tested undiluted, but following mammary infection passive protection was conferred on mice by serum diluted up to 1:32. Greatest protection was given by serum from Goat 55; serum from Goat 56 protected at 1:16, and serum from Goat 51 at 1:4. This variation in titre corresponded with the degree of severity of the mastitis which developed, and the titre of mouse-protecting antibody was highest when the alpha antitoxin content of the serum was highest.

Phagocytic power of circulating neutrophils. Although the phagocytic index varied at different samplings, there was no constant change in the index as the experimental mastitis developed. Moreover, before and after mammary inoculation the neutrophils possessed considerable phagocytic power for killed strain 201 staphylococci.

Strain BB. Goats 141 and 143 were slaughtered with gangrenous mastitis shortly after inoculation. In Goat 144, which developed mild mastitis, the only fluctuation in antibody levels detected was a slight, transient increase in the antihaemolysin content of serum. The main features of the antibody response in severe mastitis produced by this strain are compared with those of strain 201 mastitis below, and the reponse of Goat 142 is shown graphically in Fig 1.

Agglutinins and precipitins. The response was similar to that in strain 201 mastitis.

Antihaemolysins. As in mastitis produced by strain 201 there was a well-marked antihaemolysin response. This differed quantitatively from the response in strain 201 mastitis in that levels of beta antihaemolysin were in general higher than the corresponding alpha values. This could be a reflection of the somewhat lower alpha lysin production by strain BB which was noted in vitro under "Materials and Methods".

Inhibitors to coagulase and hyaluronidase. As in strain 201 infection, these antibodies were not detected in any sample of serum.

Antibody to bound coagulase. This antibody occurred only in the serum of Goat 145, at a titre of 1:4, and the level of antibody did not increase following mammary inoculation.

Inhibitors to lipase and protease. As in strain 201 mastitis the sera failed to inhibit the action of these enzymes.

Mouse-protecting antibody. This test revealed a significant difference between the two strains. The sera consistently failed to protect mice against challenge with strain BB, and even when graded doses of BB cocci down to the LD_{50} level were used as the challenge inoculum in mice, no significant difference between pre-inoculation and post-inoculation samples of serum could be demonstrated. Before inoculation, no serum protected mice against strain 201, but following mammary infection with strain BB passive protection was conferred on mice against challenge with strain 201 by serum up to a dilution of 1:4.

Phagocytic power of circulating neutrophils. Similar results were given in this test to those noted in strain 201 mastitis. Thus circulating neutrophils were capable of phagocytosing staphylococci of strain BB as efficiently as those of strain 201, and there was no constant increase in phagocytic power as mastitis developed.

DISCUSSION

A study of the characteristics of the strains of staphylococci used in Experiment 1, summarised in Table 1, failed to show differences which might account for the immunological distinction demonstrated in this experiment. It was clear that cross-immunity occurs between certain strains which are of different phage pattern, and further that immunological differences occur between strains that have other in vitro characteristics in common. It was of interest that sera from the vaccinated goats protected mice only against the strains for which a high level of immunity to mastitis was demonstrated, and not against those which produced severe mastitis in vaccinated goats. The absence of gangrene in the vaccinated goats might be interpreted as an expression of a low grade of immunity. It is also possible that immunity at this level might be demonstrated more clearly if a lower challenge dose were employed, or under field conditions in which the infecting dose of bacteria would be expected to be lower than that used in this experiment.

The result of Experiment II leads to the conclusion that strain BB (and probably N90 and S20T) differed from strains 201 and 106 in being able to produce severe mastitis in animals with a high level of alpha and beta antitoxic immunity. The fact that such antitoxic immunity is of less protective value against some strains of staphylococci than others provides a possible explanation for some of the variable results recorded in the literature on the effectiveness of

antitoxic immunity. It would appear that for strains of staphylococci such as 201, the main virulence determinant is the production of these toxins, and that the resistance of animals to such strains can be enhanced by the production of antitoxic immunity. Strains such as BB, however, seem to possess some other virulence mechanism, so that they are able to produce disease in animals with antitoxic immunity.

Experiment III failed to reveal any antigenic characteristic of strain BB which might explain its immunologically different behaviour from strain 201. The antibody response to mastitis produced by each of these strains was characterised by the development of precipitins and antihaemolysins, and an absence of the formation of antibodies to the other diffusible products of the staphylococcus, suggesting that these latter products are of limited significance in the disease. The finding that circulating neutrophils were capable of phagocytosing staphylococci was in accordance with the results of other workers (Flaum, 1938; Spink and Paine, 1940), and furthermore, the phagocytic power was not enhanced as the disease developed. It is important to emphasize that killed cocci were used in this test so that no leucocidal activity was involved. The significance of the leucocidins in staphylococcal mastitis is worthy of investigation, although it appears from the work of Gladstone and van Heyningen (1957) that the staphylococcal leucocidins which have been described are inactive against the neutrophils of ruminants.

The observation that sera from goats with severe experimental mastitis produced by strain BB did not protect mice against this strain, although the sera did protect against strain 201, could be interpreted in two ways. It may suggest that general immunity to strain BB fails to develop even when the organism is multiplying and producing lesions in the udder. Alternatively, it may be that the mouse protection test is not a satisfactorily reliable index of immunity in goats to strain BB. It would be desirable to test immunity to a second mammary challenge following a severe infection in one half of the gland, but this is unfortunately not practicable because in severe mastitis secretion by the non-infected half of the gland ceases.

CONCLUSIONS

Vaccination of goats with strain 201 adjuvant cell-toxoid conferred a high level of immunity against experimental mammary challenge with strains 201 and 106, but not against strains BB, N90 and S20T, although the production of gangrene by these strains appeared to be prevented in vaccinated goats. Immunity to mammary challenge by a particular strain was correlated with the power of the serum of the goat to protect mice against the same strain.

Vaccination of goats with strain BB adjuvant cell-toxoid failed to confer a high level of immunity against challenge with the homologous strain.

The antibody response of goats in severe mastitis produced experimentally by strain BB resembled that in severe mastitis produced by strain 201, but sera from goats experimentally infected with strain BB failed to protect mice against this strain, but did protect against strain 201.

ACKNOWLEDGMENTS

I should like to thank Dr. R. E. O. Williams for arranging for the phage pattern of the strains of staphylococci used in these experiments to be determined at the Staphylococcus Reference Laboratory, Colindale. I also wish to acknowledge the technical assistance of Miss Sylvia Spragg.

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SOME OBSERVATIONS ON AN OUTBREAK OF STAPHYLOCOCCAL MASTITIS IN A DAIRY HERD

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INTRODUCTION

Towards the end of 1956 an extensive outbreak of mastitis occurred in the Duthie Farm dairy herd of the Rowett Research Institute. Because of this, a large number of cows had to be disposed of during the following year. The disease appeared to be associated with coagulase positive staphylococci. A number of measures were taken to check the outbreak but these did not apply to the heifers in the herd. This difference should be borne in mind, but it is not proposed to discuss these measures in this paper.

The results of regular studies made on milk samples from the peak of the outbreak onwards have now been examined alongside the clinical results in an attempt to gain some insight on an occurrence of great economic importance. There have been similar outbreaks of mastitis in other dairy herds in the North-East of Scotland (Shanks, 1959).

METHODS

Bacteriological tests. Just prior to the morning milking on the first Monday of each calendar month, 25 ml. of milk were taken as aseptically as possible from each animal, in equal quantities from each quarter. Because fresh milk has been shown to cause clumping of staphylococci (Smith, 1957), the samples of milk were centrifuged at 2000 to 2500 r.p.m. in an M.S.E. minor centrifuge for 10 minutes. The deposit was made up to 0·5 ml. with the supernatant milk from the same sample, and emulsified; 0.01 of the emulsion representing the deposit from 0.5 ml. of milk was spread on a 5 per cent fresh sheep blood agar plate, the base of which was a tryptic digest of beef muscle. The plates were examined after 24 hours and again after 48 hours, and individual counts were made of all haemolytic and non-haemolytic staphylococci. Staphylococci were identified by their morphology and ability to grow in 10 per cent NaCl broth. The coagulase test was carried out on plates of bovine fibrinogen agar (Boake, 1956; Lack, 1957).

The Whiteside test modification No. 2. This was carried out immediately prior to the afternoon milking, using a solution of 0·5 per cent Delsanex in 1·5 per cent NaOH. A Perspex paddle was constructed by glueing together two sheets of 9″ by 9″ Perspex, one $\frac{1}{2}$″ thick, which had four 3″ holes bored through it, and the other $\frac{1}{8}$″ thick as the base. A Duralumin handle was added. Approximately 3 ml. of milk from each quarter were collected in the cups thus produced, 3 ml. of reagent were added from a pipette and mixed by a rotational movement. Positive results were scored as +, + +, or + + + according to the strength of the reaction.

RESULTS

The Herd

Composition. The greater part of the herd consists of home-bred pedigree Ayrshires, but there are also a number of sets of twin animals of various breeds bought as young calves. There are normally about 80 animals in milk but the number varies somewhat with the time of year, and dropped well below this figure during the period of the outbreak due to an increased disposal rate and lack of heifer replacements.

Management. The herd is usually housed day and night from November to April and only at night during May and October, but these dates are varied slightly from year to year according to the prevailing weather. No bulls are kept; all breeding is by artificial insemination.

There are two byres, each with two rows of 22 stands facing a central feeding passage. The byres are separated by a feed store and calving pens, and each byre has its own staff who do all the work required, including milking. This is carried out in a joint milking parlour which runs along the ends of the byres, feed store and calving pens. The milking machine is an eight point Alfa Laval combine recorder releaser type. Four of the milking points are allocated to each byre.

When they are indoors the animals are fed on a conventional basal diet of roots and silage, with concentrate supplements according to their level of production. Out-of-doors they graze ley pasture, with limited concentrate supplements.

Heifer management. There is no contact between the milking herd and young stock, as each have separate buildings, staff and grazing. Heifers calving from May to October calve at pasture, still without contact with the dairy herd. Those due to calve between November and April are taken into the byre with the milking herd in November, and just prior to calving they are removed to a separate calving pen. Since Autumn 1957 it has been the practice for all animals calving to suckle their calves for four days and for surplus milk to be removed twice a day by machine.

The Mastitis Outbreak and its Treatment

Occasional cases of mastitis associated with staphylococci or streptococci had always occurred in this herd and it had long been the practice to use penicillin in their treatment. Towards the end of 1956 there was a sharp increase in the number of cases from which coagulase positive staphylococci were isolated. At the beginning of 1957 it was found that coagulase positive staphylococci isolated from milk from mastitis cases were resistant to penicillin but not to chlortetracycline or to chloramphenicol, and from that time cases were treated with intramammary chlortetracycline. In February 1957 all quarters of all lactating animals in the herd were treated with intra-

mammary chlortetracycline. The outbreak continued without any apparent check, and in March and April 1957 large numbers of cows were sold in an attempt to rid the herd of all severely affected animals. Various other measures were tried including the use of individual udder cloths, milking clinical cases last, and steam sterilization of the milking machine lines twice daily after milking. Following this a number of cases were treated with chloramphenicol, again with no appreciable effect on the number of new cases arising. At this point help was sought from outside the Institute and subsequent treatment was based on the recommendations received. From June 1957 all clinical cases were treated with intramammary novobiacin, the teats of all cows and the portion of the udder touched by the teat cups were dressed with antiseptic cream once daily after milking (for the first month 1 per cent Hibitane Cream,* was used, and from then on Dequadin Cream†); at the same time the use of a disinfectant (Chloros‡) in the water for washing udders and for dipping teat dusters was discontinued. The milking machine pressure was adjusted to 15″. Steaming after the afternoon milking ceased at the beginning of August 1959. Every attempt was made to keep the milking time as short as possible. From August 1957 cows were treated with intramammary novobiacin when they were dried off.

The outbreak continued without any appreciable improvement up to November, 1957. In December there was a sudden reduction in the number of clinical cases, and since then there has only been an occasional case of mastitis in the herd.

Records were kept of the incidence of the disease in the milking herd and in heifers at calving. In addition, monthly bacteriological examinations were made of milk samples from all lactating cows from July 1957 to April 1959 (except in September 1958) and again in July, October and December, 1959. The Whiteside test, modification No. 2 of Schalm and Noorlander (1957), was carried out for all lactating quarters on the same day as the monthly bacteriological test.

Incidence of Clinical Cases of Mastitis

An animal was taken to be a clinical case of mastitis if its milk was macroscopically abnormal and unfit for sale for two or more successive milkings. Signs of inflammation, induration and anorexia were sometimes present, singly or in combination, and in varying degree.

Over the period from April, 1957 to December, 1959, there were 214 clinical cases of mastitis from which coagulase positive staphylococci were isolated. The incidence of the disease in each month of this period is shown in Fig. 1A, where the number of days on which each cow showed clinical symptoms, totalled over all the cows in the

* Pharmaceutical Division, Imperial Chemical Industries, Ltd.
† Allen and Hanbury Ltd.
‡Pharmaceutical Division, Imperial Chemical Industries, Ltd.

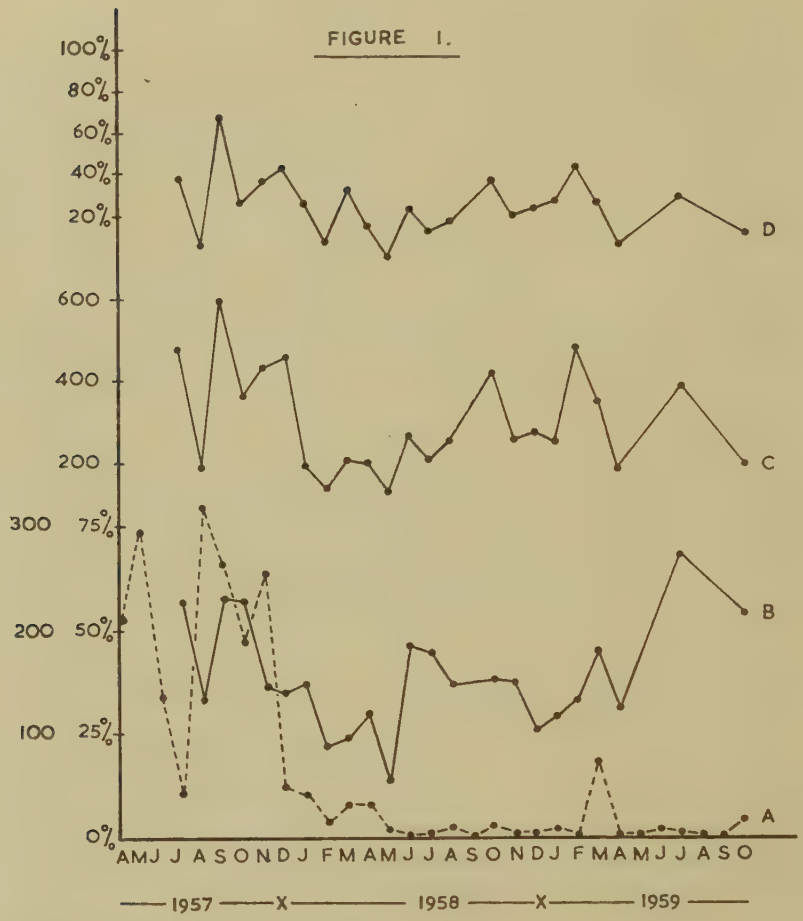

Fig. 1. (A) Cow days of mastitis/month.
(B) % of cows carrying coagulase positive staphylococci at each monthly test.
(C) Mean count of coagulase positive staphylococci/ml of positive samples.
(D) % of milk samples containing coagulase positive staphylococci that were uncountable.

milking herd, is plotted for each month. The total number of animals in the milking herd within this period of 33 months was 162. Out of 90 which were in the herd before August 1957, 76 suffered clinical attacks of mastitis, most of them on more than one occasion. Table 1 shows how many attacks were experienced by these animals, and also by the 72 animals added to the herd from August 1957 onwards.

The outbreak seriously affected animals coming into milk for the first time. Table 2 shows the numbers and percentages of heifers calving down with mastitis at different times of year, according to whether they calved indoors or at pasture.

TABLE 1

NO. OF CLINICAL ATTACKS OF MASTITIS IN EACH ANIMAL FROM APRIL 1957 TO DECEMBER 1959

No. of attacks (N)	*No. of animals attacked N times*	
	In herd before August 1957	*Added to hard later*
0	14	60
1	23	8
2	25	4
3	11	0
4	7	0
5	4	0
6	2	0
7	2	0
8	1	0
9	0	0
10	1	0
	90	72

Loss of Animals from the Herd

The numbers of animals which were put out of the herd because of severe attacks of mastitis between 1957 and 1959 are shown in Table 3. Disposals were particularly heavy in March and April 1957, when a special attempt was made to rid the herd of all severely affected animals.

Milk Yields and Loss of Milk

Mean milk yields for completed lactations are taken from the figures of the Scottish Milk Records Association (Table 4). They are given separately according to the six-monthly periods in which each animal calved down, and are separated for cows and for heifers. The quantity of milk discarded as unfit for sale each month from April 1957 to December 1959 is shown in Table 5.

Bacteriological Studies of Milk Samples

Considerable numbers of coagulase negative staphylococci were isolated, but the results presented here relate only to the coagulase

TABLE 2

NUMBER AND PERCENTAGES OF HEIFERS CALVING DOWN WITH MASTITIS ACCORDING TO PLACE AND MONTH OF CALVING

	Byre				*Pasture*						*Byre*		
Month	*J*	*F*	*M*	*A*	*M*	*J*	*J*	*A*	*S*	*O*	*N*	*D*	*Total*
1956													
Number calving	1	1	1	1	2	9	3	2	1	9	7	0	
Mastitis	0	0	0	0	0	0	0	0	1	1	4	0	
% Mastitis			0				8				57		16
1957													
Number calving	7	4	7	1	1	0	8	3	1	5	16	4	
Mastitis	6	3	6	1	1	0	3	2	0	0	6	1	
% Mastitis			84				33				35		51
1958													
Number calving	1	1	8	2	0	2	0	0	2	5	4	3	
Mastitis	0	0	3	0	0	0	0	0	0	0	0	0	
% Mastitis			25				0				0		11
1959													
Number calving	4	2	2	1	2	0	1	5	1	1	2	1	
Mastitis	0	0	0	0	0	0	0	0	0	0	0	0	
% Mastitis			0				0				0		0

positive staphylococci. Many different types of these could be picked out by differences in colony size, pigment, and the diameter of the zone of α-haemolysin and of β-haemolysin. Of all the isolates examined 94·4 per cent produced α- and β-haemolysins, 1·8 per cent produced α only, 2·5 per cent produced β only, and 1·3 per cent produced no haemolysis. All the coagulase positive staphylococci tested in 1957 were resistant to penicillin*. After the end of the outbreak, no more antibiotic testing was done until Spring 1960 and then occasional penicillin sensitive coagulase positive staphylococci were found.

The numbers of cows in whose milk coagulase positive staphylococci were found each month, expressed as percentages of the numbers of cows tested each month, are given in Fig. 1B, and the numbers with more than 800 of these organisms per ml. of milk, again as percentages to the same base, are given in Fig. 1D. An approximate figure for the average count of staphylococci per ml. of milk in the positive samples is given in Fig. 1C. By the method used the maximum count obtainable was 800/ml., and an arbitrary figure of 100/ml. has

* Sentest Tablets No. 2, penicillin 2·5 I.U., Evans Medical Supplies.

been taken for all samples which were uncountable, so these last results are not exact. The incidence of staphylococci was anything but random. They were almost always found in tests on some cows and rarely in tests on others. Certain cows gave positive tests in one

TABLE 3

ANIMALS DISPOSED OF BECAUSE OF MASTITIS

Month	*No. disposed of*		
	1957	*1958*	*1959*
January	1	0	0
February	2	0	0
March	16	0	1
April	15	0	0
May	5	0	0
June	3	0	0
July	2	0	0
August	2	1	0
September	2	0	1
October	1	1	0
November	5	0	1
December	3	0	1
Annual totals	57	2	4

TABLE 4

MEAN MILK YIELDS IN COMPLETED LACTATIONS

Scottish Milk Records Association figures

Dates of calving	*No. of cows*	*Mean yield (lb)*	*No. of heifers*	*Mean yield (lb)*
1956 January-June	62	7790	14	5694
July-December	31	6947	20	6288
1957 January-June	36	7111	14	3604
July-December	30	7368	31	6419
1958 January-June	33	8194	13	6042
July-December	37	9574	14	6890

TABLE 5

MILK DISCARDED AS UNFIT FOR SALE

Month	*lb. Milk*		
	1957	*1958*	*1959*
January	*	950	130
February	*	610	60
March	*	1120	2800
April	5170	1830	0
May	8570	100	0
June	4780	110	100
July	4940	0	210
August	12180	210	0
September	8090	210	110
October	5130	180	560
November	3050	0	640
December	770	120	1390
Average/month	5850	450	500

* Figures not available

lactation but not in a preceding or a following one. The distribution of incidence by animals is shown in Table 6, separately for 79 animals which were in the herd before August 1957 and for 49 animals which were added thereafter, when all the control measures had been put into effect. Animals tested fewer than five times have been excluded from this table. It may also be noted that on the first test carried out on all the heifers joining the herd from August 1957 onwards, 30 per cent showed coagulase positive staphylococci in the milk, a figure not substantially lower than the herd average.

Whiteside Test (Modification No. 2)

The results of these tests are shown in Fig. 2 which plots monthly figures for—

(a) % of quarters positive. (All grades of severity)
(b) % of quarters ++
(c) % of quarters +++
(d) % of cows having one or more positive quarters.

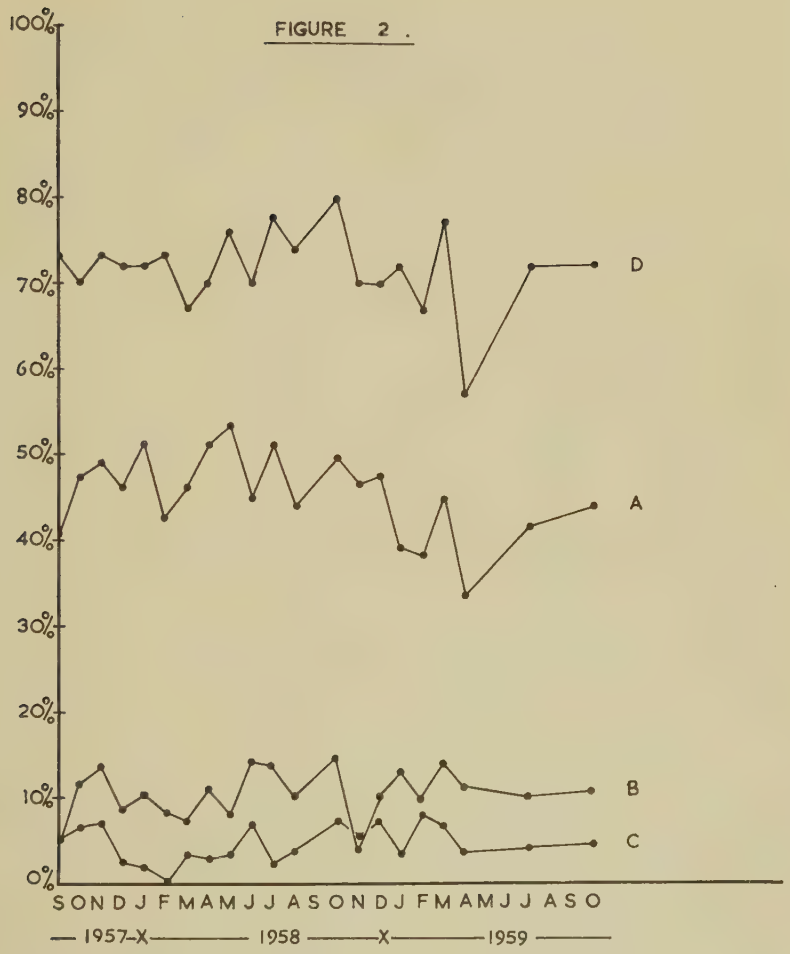

Fig. 2. Whiteside test modification No. 2.
(A) % of quarters positive, all grades at each monthly test.
(B) % of quarters ++ at each monthly test.
(C) % of quarters +++ ,, ,, ,,
(D) % of cows having one or more positive quarters at each monthly test.

Bacteriological and Whiteside Results in the Light of Subsequent Disposals from the Herd

Table 7 shows how the results of these tests, limited to the period July 1957 to June 1958, compare between cows which were still in the herd in July 1959 and cows which had been disposed of by that time. Results from twin cattle have been excluded in order to cut down the number of variable factors concerned in this internal comparison.

TABLE 6

RECOVERY OF COAGULASE POSITIVE STAPHYLOCOCCI

Distribution of percentages of tests at which each animal's milk yielded coagulase positive staphylococci; July 1957—December 1959. Percentage for each animal based on at least 5 tests

% of positive tests	*Numbers of animals*	
	In herd before August, 1957	*Added to herd later*
0	1	7
1–25	20	22
26–50	34	12
51–75	15	7
76–100	9	1
	79	49

TABLE 7

TESTS ON MILK RELATED TO LATER DISPOSALS

Group of cows			*A**	*B**	*C**	*D**	*Total*
No. in group			46	8	13	12	79
Average no. present per test			27·5	3·5	6·8	6·4	44·2
Percentages of milk samples	Containing *H. Strept.*		19	18	23	15	19
	Containing *Staph.*	1–10 per ml.	6	3	12	6	7
		11–100 per ml.	17	18	11	15	16
		101–800 per ml.	3	15	4	5	4
		Uncountable	11	23	6	14	12
Whiteside test Percentages of quarters	—ve		58	26	54	55	55
	+		31	36	31	30	31
	++		8	22	12	10	10
	+++		3	16	3	6	4
Clinical symptoms noted (days/animal/month)			1·0	7·8	1·0	1·0	1·5

*A Still in herd 1.7.59
B Disposed of (mastitis)
C Disposed of (infertile)
D Disposed of (other reasons)

Out of a total of 79 cows tested at least once between July 1957 and June 1958.

DISCUSSION

Since these observations do not describe a controlled experiment, they can not be expected to give rise to hard and fast conclusions, and it is not proposed to discuss to what extent the outbreak was affected by the various measures taken. Nevertheless we think there are a number of points of interest in the data.

The drop in the incidence of clinical cases after November 1957 (Fig. 1A) was not accompanied by any appreciable fall in the incidence of coagulase positive staphylococci in milk samples (Figs. 1B, C and D) or by any outstanding change in the Whiteside test results (Fig. 2). The former observation must throw some doubt on whether the coagulase positive staphylococci were the actual cause of the disease. Table 7 confirms that there was in fact some relationship between the disease as clinically defined and some of the milk test results, including the incidence of high counts of coagulase positive staphylococci but not the incidence of streptococci. The evidence suggests either that there is some unknown set of circumstances which, in conjunction with the presence of staphylococci, together cause clinical outbreaks of the disease, or alternatively, that only particular strains of the organism can produce clinical symptoms. It is not possible to say whether the organisms isolated during the clinical outbreak were of the same strains as those isolated afterwards.

The question of immunity engendered by an attack of the disease is raised by the data in Table 1. Of the 88 animals affected, 57 were clinical cases more than once, and 17 were clinical cases four times or more. Clearly either an attack did not confer immunity or else a number of strains of bacteria were concerned and did not cross protect.

The incidence of the disease among the heifers is of some interest. Comparison of the percentages of heifers calving with mastitis (Table 2) with the overall incidence of clinical mastitis (Fig. 1A), shows that the outbreak followed much the same course and at the same time amongst both heifers at calving and dairy cows. This is difficult to explain, since either the preventive treatment was not applied or in fact was not applicable to the heifers. The system of management kept young stock strictly apart from the dairy cows, except that the heifers due to calve in the winter months were brought into the byre with the dairy herd in November. These animals could not have been infected from the milking machine, and byre hygiene could not have exerted any direct influence on the decline of cases. The coincidence may indicate another gap in our present knowledge of the origin and spread of these outbreaks.

The outbreak clearly had a markedly depressing effect on the mean milk yields in completed lactations (Table 4), and the fall appeared to be somewhat greater in the heifers than in the dairy cows.

It is hoped that it may be possible to collect more useful information in future on the basis of work which is now in progress, aimed at

discovering whether strains of staphylococci producing clinical cases are serologically identical to those found in the milk in the absence of clinical symptoms.

CONCLUSIONS

Observations are presented on an extensive outbreak of mastitis associated with coagulase positive staphylococci in a dairy herd.

The levels of coagulase positive staphylococci in milk samples and the results of a modified Whiteside test remained relatively constant although the incidence of clinical cases dropped markedly after the outbreak passed its peak. Procedures adopted to control the outbreak did not apply to animals at first calving but clinical cases amongst these also declined.

ACKNOWLEDGMENTS

We wish to express our thanks to the following: Mr D. Davidson and Mr E. A. S. Rattray for technical assistance; Mr W. Thomson, Head of the Duthie Experimental Stock Farm; Mr J. B. Gunn, Assistant, and also the byre staff of the farm, for information and practical assistance; Mr D. A. McKenzie, N. of Scot. Coll. of Agriculture for initial supplies of Delsanex; and Delsanex (Scotland) Ltd., for a further free supply of Delsanex.

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EXPERIMENTAL TRANSMISSION OF SCRAPIE TO GOATS AND SHEEP BY THE ORAL ROUTE

By

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INTRODUCTION

It is now well established that scrapie can be experimentally transmitted to sheep and goats by intracerebral, epidural, intraocular or subcutaneous inoculation of suitable material. However, very few observations have been made on the possibility of transmitting the disease by mouth. Thus, it would appear from the literature that the only deliberate attempts at oral transmission have been those of M'Fadyean (1918) who failed to cause the disease in one sheep given a drench of "part of the contents of the small intestine" of a scrapie-affected sheep, and of Stamp, Brotherston, Zlotnik, Mackay and Smith (1959) who failed to produce the disease in any of seven day-old Cheviot lambs dosed orally with 5 ml. of a suspension of scrapie sheep brain. M'Fadyean's experimental animal was observed for 29 months, and those of Stamp *et al.* for 12 months. In addition to these reports, Greig (1940) examined earlier conflicting evidence that the disease could be transmitted to suckling lambs through the milk of scrapie-affected ewes and concluded that this possibility remained undetermined.

In view of the paucity of information on the possibility of transmitting scrapie by mouth, and because of the important bearing that this information might have in explaining the method of spread of the disease under field conditions, the observations described in this paper were made. Experiments were carried out to examine further the possibility of transmitting scrapie orally to goats and sheep, and attempts were also made to transmit the disease to goats by intracerebral inoculation of certain secretions and excretions from a scrapie-affected goat.

MATERIALS AND METHODS

Scrapie material used. The "strain" of scrapie, described by Pattison, Gordon and Millson (1959), had been maintained at Compton by serial passage through sheep and goats. The scrapie material used for oral dosing of sheep had been passaged only through sheep, and that used for goats had been passaged three times through goats.

Oral dosing of goats. Two three-month-old castrated male goats from the Compton flock were given by drenching bottle approximately 100 ml. of a 10 per cent suspension of goat brain in normal saline prepared from a pool of three deep-frozen brains of scrapie-affected goats destroyed three days previously.

Oral dosing of sheep. Each of 50 sheep was drenched with approximately 100 ml. of a 10 per cent suspension in normal saline of scrapie brain prepared from a pool of 15 deep-frozen brains of South Scotland Cheviot sheep that were clinically affected with the disease when slaughtered between two days and six months before preparation of the suspension. Each of a further 46 sheep received approximately 100 ml. orally of a 10 per cent saline suspension of normal brain made from a pool of one Dorset Down brain, one Clun Forest, one Swaledale and one Dalesbred. The sheep used ranged in age from about six to about 11 months, and the breeds are detailed in Table 1.

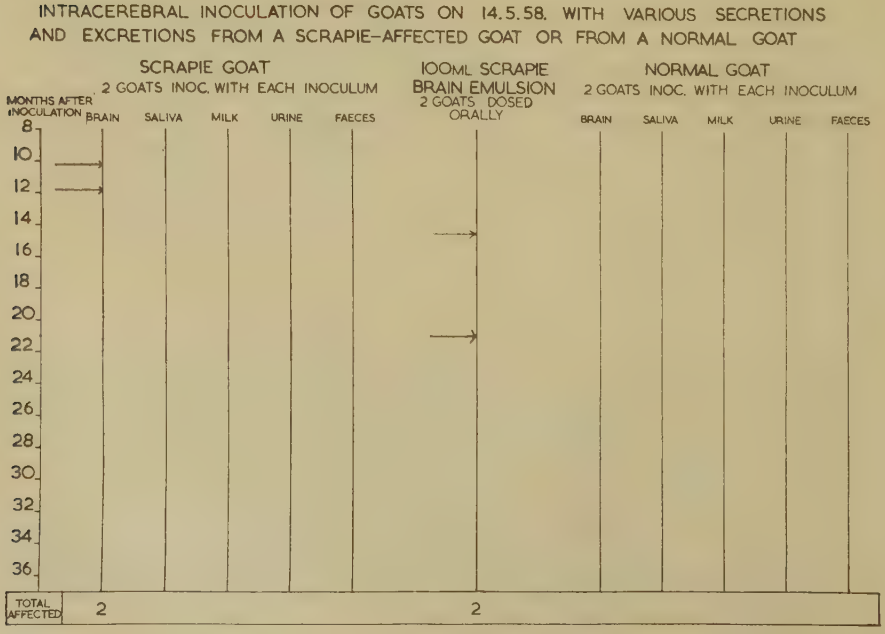
Intracerebral inoculation of goats. Pairs of three-month-old goats from the Compton flock were inoculated intracerebrally with 1.0 ml. of the following materials obtained either from a scrapie-affected goat or from a normal goat: (a) brain—the supernatant of a lightly centrifuged 10 per cent. suspension in normal saline (b) saliva—collected as it dripped from the mouth under general Nembutal anaesthesia and then passed once through a Seitz EK sterilising pad (c) milk—whole milk drawn from the udder with aseptic precautions (d) urine—obtained by puncture through the bladder wall after death and passed once through a Seitz EK sterilising pad (e) faeces—a 10 per cent suspension in normal saline filtered through muslin, lightly centrifuged, and then passed once through a Seitz EK sterilising pad.

RESULTS

Goats

The observations summarised in Fig. 1 were made in order to examine the possibility that the scrapie agent may be (a) voided from

Fig. 1



an affected animal in some secretion or excretion (b) absorbed by an in-contact animal by the oral route. From the results given in the figure it will be seen that the only cases of scrapie that have occurred to the time of writing (29 months after the start of the experiment) have been (a) the two animals inoculated intracerebrally with scrapie goat brain, at approximately 10½ and 12 months, respectively, after inoculation, and (b) the two animals dosed orally with scrapie goat

TABLE 1

OCCURRENCE OF SCRAPIE IN SHEEP DOSED ORALLY WITH SCRAPIE SHEEP BRAIN

Breed	*No. dosed orally with normal brain**	*No. dosed orally with scrapie brain*	*Cases of scrapie*	*Incubation period: months*
Clun Forest	1	1	0	—
Dalesbred	0	1	1	7½
Derbyshire Gritstone	3	3	1	5½
Dorset Down	1	2	0	—
Dorset Horn	3	4	0	—
Exmoor Horn	2	1	0	—
Hampshire Down	3	2	0	—
Leicester	1	1	0	—
North Scotland Cheviot	7	8	0	—
Romney Marsh	3	3	0	—
Ryeland	2	2	0	—
Scottish Blackface	1	1	0	—
Shropshire	1	1	0	—
Southdown	1	2	0	—
South Devon	2	2	0	—
South Scotland Cheviot	2	3	3	9:10:10:
Swaledale	4	4	1	10½
Suffolk	3	3	0	—
Welsh Mountain	2	2	0	—
Wiltshire Horn	4	4	1	7½
Total	46	50	7	—

* No case of scrapie occurred in these animals.

brain, at approximately 15 and 21 months, respectively after dosing. The clinical appearance of the affected animals was typical of scrapie in the goat (see Pattison *et al.*, 1959; Pattison and Millson, 1961), and histological examination of their brains confirmed the clinical diagnosis. Observation of the surviving animals in this experiment continues.

Sheep

In view of this successful oral transmission of scrapie to goats, it appeared desirable to make further observations using sheep. Details of the animals used are given in Table 1. It will be seen that to the time of writing (11 months after dosing) seven cases of scrapie have occurred in animals dosed with scrapie brain, but none in animals dosed with normal brain. The incubation period ranged from five-and-a-half to 10½ months, and the breeds involved were Dalesbred, Derbyshire Gritstone, South Scotland Cheviot, Swaledale and Wiltshire Horn. Clinical symptoms of the disease included rubbing of the rump, nibbling of the legs, and unsteadiness of gait, and were typical of the "itching" form of scrapie. Observation of the surviving animals in this experiment continues.

DISCUSSION

Earlier investigators of scrapie in Britain (Stockman, 1913; M'Fadyean, 1918) produced impressive field evidence to indicate that scrapie could spread among sheep at pasture in the absence of any breeding contact. The indications were that in certain circumstances pasture could be contaminated by scrapie-affected sheep and that normal sheep subsequently grazed on this pasture might contract the disease. Apparently a very considerable time might elapse between pasture contamination and the appearance of scrapie in sheep grazed thereon. Greig (1940) examined this possibility experimentally and produced evidence that appeared to confirm these observations. While recognising the possibility that the disease could spread among sheep at pasture, M'Fadyean emphasised that all his attempts to transmit scrapie from diseased to healthy sheep housed indoors had failed, and this experience has been shared by others (Stamp *et al.*, 1959; Pattison and Millson, 1961) who have tried without success to spread scrapie by direct contact among housed animals.

During the course of a large-scale scrapie transmission experiment in which scrapie-inoculated and control animals were run together as a flock, Gordon (1957) recorded that the disease appeared in one of 30 non-inoculated sheep, and in five of 60 sheep inoculated subcutaneously with sterile saline and broth. This gave an overall occurrence of six cases of scrapie out of 90 sheep that had not been inoculated with scrapie material. The first of these cases occurred 29 months, and the last 42 months, after the other sheep in the flock had

been inoculated with scrapie material. A possible explanation for these observations was that the disease had spread by contact from affected to healthy sheep.

It would appear, therefore, that in certain circumstances scrapie may spread among animals at pasture. On the other hand the fact that extensive attempts to pass it from diseased to healthy sheep or goats kept indoors have been unsuccessful, suggests that some factor important in the spread of the disease is present out-of-doors but absent, or of reduced effectiveness, indoors. Without attempting to postulate what this factor may be, it might reasonably be suggested that if the scrapie agent is voided by an affected animal the fact that the disease can be reproduced by oral dosing with scrapie brain might offer an explanation for pasture transmission.

The negative results reported here of attempts to produce scrapie in goats by intracerebral inoculation of saliva, milk, urine or faeces from a scrapie-affected goat must not be taken to mean that the scrapie agent was absent from these inocula. Many further observations are necessary before that conclusion could be reached. It is possible, for example, that the agent was present in too small an amount to create the disease, or that Seitz filtration reduced the titre to a non-infective level. But the fact that the agent can create the disease by the oral route indicates that it may be able to survive in the alimentary tract, and it may yet be possible to demonstrate its presence in the excretions of affected animals.

It is of interest that the number of cases of scrapie produced in sheep by the oral route (seven out of 50 within 11 months) is comparable with the number that might be expected to follow intracerebral or subcutaneous inoculation of a similar group of sheep. Further, an incubation period ranging from five-and-a-half to 10½ months is similar to that observed following direct inoculation of scrapie brain suspension.

CONCLUSIONS

Scrapie occurred in both of two goats dosed orally with 100 ml. of a suspension of scrapie goat brain, and in seven of 50 sheep dosed orally with the same volume of scrapie sheep brain.

During an observation period of 29 months, scrapie did not occur in goats inoculated intracerebrally with unfiltered milk or with filtered saliva, urine or faeces from a scrapie-affected goat. These findings do not neccessarily mean that the scrapie agent is absent from these secretions and excretions and further experiments with these materials are needed.

It is suggested that infection by mouth might explain the observation of earlier authors that, without breeding contact, scrapie may sometimes spread from sheep to sheep at pasture.

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CONGENITAL MYOPATHY IN LAMBS

By

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INTRODUCTION

Stamp (1960) has recently described the occurrence of muscular dystrophy and neonatal mortality in a flock of Suffolk sheep maintained by Moredun Institute. The purpose of the present communication is to describe congenital abnormalities which have been observed in this flock and which are also associated with myopathy.

OBSERVATIONS

Clinical Features

The Suffolk flock referred to above was formed in 1955, the first lambcrop being born in 1956. In this and in each subsequent year a number of lambs have shown at birth two types of abnormality. In the first there was an inability to raise the head into its normal position (Figs. 1 and 2). This condition varied in its severity, milder cases carrying their heads so that neck and back formed a straight line, while in the most severe cases their noses were almost touching the ground. Deviation of the neck to one side (scoliosis) was never seen. Severely affected lambs had considerable difficulty in reaching the dams' teats. The majority of these lambs made a complete spontaneous recovery within a few months, only a few remaining permanently affected and unable to elevate the head and neck above the horizontal.

In the second there was flexion of the lower limb joints, usually affecting the fetlocks, but occasionally involving the carpal joints. In these cases the forelimbs were always affected, the hindlimbs less frequently. In some lambs these joints could be extended at birth by the use of slight force, flexing again when pressure was released, but in a few cases even this was not possible. When standing and walking, the forelimbs were held further forward than normal and the weight of the lamb was borne on the anterior aspect of the fetlock joint. The skin over this part therefore became denuded of hair and abraded. The ewes carrying lambs with this type of abnormality invariably required assistance at parturition. Every case of this type made a complete spontaneous recovery, the time taken varying from a week to three months.

A small number of lambs showed both types of abnormality. Details were obtained in 1958, 1959 and 1960 of the numbers of lambs affected (Table 1).

TABLE 1

Year	No. of lambs born	Affected lambs			
		Total numbers	Number with		
			Head down	Flexed joints	Both abnormalities
1958	199	11	5	5	1
1959	185	7	3	4	—
1960	135	10	7	2	1

Pathology

Since this flock of sheep is being used to investigate the possibility of genetic factors in the etiology of scrapie, we have not been able to sacrifice selected lambs to obtain material for a comprehensive study of these abnormalities. The following observations on the morbid anatomy and histopathology are based on a small number of cases which became available as a result of culling and deaths from intercurrent disease. In all, material was obtained from 4 lambs varying from 1 day to 1 month of age, and from 3 older animals between 1 and 3½ years of age which had been affected since birth.

Anatomical findings in lambs. The changes which were observed were as follows:

Lamb A (ten days old). There was partial flexion of the carpal joints. Small bilaterally symmetrical chalky-white streaks were present on the muscles of the lateral aspect of the thighs close to the stifle joints. No other macroscopic lesions were noted.

Lamb B (one day old). The head and neck were depressed. Pallor and oedema of the muscles overlying the first few cervical vertebrae were noted.

Lamb C (one month old). The head and neck were depressed. Bilaterally symmetrical chalky-white streaks and patches were present on the scalenus, subscapular and brachialis muscles.

Lamb D (three days old). The head and neck were depressed and there was partial flexion of the fore fetlock joints. Chalky-white streaks were found on one scalenus muscle only.

Histological findings in lambs. The microscopical changes were as follows:

In lambs A, C and D all macroscopically affected muscles showed lesions of muscular dystrophy, with necrosis and calcification of muscle fibres and some attempts at regeneration.

In lamb B the muscles of the dorsal cervical region showed necrosis of their fibres, which were separated by oedema fluid containing numbers of histiocytes.

Anatomical findings in older sheep. A ewe hogg, aged one year, had been unable to raise its head into a normal position since birth, and

had a tendency to grunt and appear distressed when made to move. Throughout its life symptoms of respiratory distress on exertion became increasingly evident, latterly even slight movement producing severe dyspnoea. Eventually the stress of driving it to a nearby field proved fatal. Post-mortem examination showed intense generalised venous congestion and marked subcutaneous oedema of the head and neck, extending to the entrance to the chest. Oedema of the larynx was also found. The cause of these changes appeared to be a deformity of the lower neck which had markedly restricted the size of the entrance to the thorax. Death was apparently due to obstruction of the venous return from the head and neck and resultant laryngeal oedema. Further examination showed bilaterally symmetrical fibrous lesions in the longissimus cervicis muscles overlying vertebrae C6, C7 and T1. Dissection of the vertebrae in this region showed the bodies of the C7 and T1 vertebrae to be wedge-shaped, the points of the wedges being ventral, this deformity exaggerating the normal ventral convexity of the latter part of the cervical series.

A ewe hogg, aged one year three months (Fig. 3), was unable to raise its head into a normal position since birth. The cause of death was a peracute enteritis. There was no evidence of circulatory obstruction at the entrance to the chest as in the previous ewe, but again both longissimus cervicis muscles showed fibrous replacement of the parts overlying the C6 and C7. Removal of the vertebral arches showed pronounced kyphosis at C7 and T1 (Fig. 4), which appeared likely to have caused some compression of the spinal cord. Sectioning of the cervical and first few thoracic vertebrae showed that the kyphosis was produced by the wedge-shaping of C7 and T1 as in the previous animal (Fig. 5).

A wether, aged 3½ years, had been unable to raise its head above the level of its back since birth. It developed typical symptoms of scrapie, and was therefore killed. The longissimus cervicis muscles showed symmetrical lesions overlying C6 and C7 consisting of marked fibrosis, the fibrous tissue being intermingled with considerable amounts of fat and containing a few surviving bundles of muscle fibres (Fig. 6). Similar but less marked lesions were present in both serratus cervicis muscles. Removal of the vertebral arches revealed an elevation of the spinal cord over the T1 with flattening of the ventral surface of the cord at this site. T1 was displaced dorsally, forming steps in the floor of the canal at its anterior and posterior extremities. Sectioning of the vertebrae showed wedging of T1 with associated lesser deformation of C7 and T2 (Fig. 6).

Histological findings in older sheep. In all three sheep the changes were essentially similar. The principal features of the lesions in the longissimus cervicis muscles were atrophy and replacement by fibrous tissue and fat cells. Necrosis with fragmentation of the muscle fibres was also seen, but less commonly (Fig. 7). The central part of the lesion consisted almost entirely of bands of fibrous tissue running in

the same direction as the muscle fibres, with groups of fat cells scattered unevenly between them (Fig. 8). Staining by phosphotungstic acid haematoxylin revealed remnants of muscle fibres completely enveloped by fibrous tissue and showing atrophic changes, but with striations still remaining visible in most of them (Figs. 8, 9 and 10). Towards the periphery of the lesion the amounts of fibrous tissue decreased, while the number of fat cells was correspondingly greater. Muscle fibres were seen showing loss of sarcoplasm, the collapsed sarcolemmal sheaths remaining. A few multinucleate muscle buds were also present.

In the first hogg, in addition to skeletal muscle changes, areas of degenerative change in the subendocardium of both ventricles of the heart were seen, while associated Purkinje fibres were similarly affected (Fig. 11). A section of the bodies of the affected vertebrae of the second sheep showed no evidence of fractures, either recent or old-standing (Fig. 12).

Examination of the central nervous system of these sheep has shown no abnormalities which could be attributed to or associated with the vertebral lesions.

DISCUSSION

On account of the lack of available material it is not possible to say more regarding the flexed limb-joint cases than to point out that myopathy of the vitamin E deficiency type is apparently associated with the condition.

Inability to raise the head in newborn lambs has been described by Hartley and Dodd (1957), who found lesions in the muscles overlying the cervical vertebrae, with necrosis, oedema and subsequent rapid calcification of muscle fibres. They named the condition congenital muscular dystrophy. Surviving lambs made a complete recovery in a few days and none showed permanent deformity. Congenital myopathy in lambs has also been recorded by Muth (1955) and Grant, Hartley and Drake (1960), the emphasis in both cases being on myocardial lesions, although skeletal muscle was also affected. The histological findings in the young lambs which are described in this paper are similar to those described by Hartley and Dodd (1957) and therefore closely resemble the myopathy due to deficiency of vitamin E or of selenium.

Roberts (1929) and Middleton (1934) described a condition seen in Welsh Mountain lambs, characterised clinically by fibrous contractures of limb joints at birth and pathologically by degeneration of muscle and replacement by fat and fibrous tissue. This was named 'Myodystrophia foetalis deformans' and Roberts stated that the condition was without doubt inherited and due to a simple autosomal recessive factor. Affected lambs were usually stillborn, or died soon after birth. Miller (1960), who carried out the pathological examination of these lambs, mentions that, although the principal feature was

fibrous contracture of the limbs, a proportion of these Welsh Mountain lambs also showed bilateral cervical muscle degeneration.

Morley (1954) reported a condition found in lambs dying shortly after birth in New South Wales, characterised by rigid flexures of the limbs, which he named 'Lethal myodystrophia'. This was stated to be genetic in origin and due to an autosomal recessive factor. Histological examination of skeletal muscle showed dystrophic lesions which must have occured in utero some considerable time prior to birth, since fibrous replacement of affected muscles had occurred.

Whittem (1957) has described a congenital abnormality in calves (arthrogryposis) associated with the absence of ventral horn cells in regions of the spinal cord, causing atrophy, and fatty and fibrous replacement of muscles supplied by them. Clinically this was characterised by contractures of the limbs, and in a few cases deformities of the vertebral column such as torticollis, where atrophy of the neck muscles was occasionally found. No conclusion was reached regarding its etiology.

The number of sires used in the Moredun flock has made it difficult to establish whether or not a genetic factor is involved. No abnormalities have been observed in the brain or spinal cord of the affected animals.

The cause of the neck deformity described in this paper would seem to be degenerative lesions, usually bilaterally symmetrical, which are present at birth in the neck muscles, particularly the longissimus cervicis. How they arise and at what time before birth are not known, although by comparison with the cases of Roberts (1929) and Morley (1954) it would seem clear that the onset of muscle degeneration in the Moredun lambs occurs as a general rule close to the time of parturition, since complete recovery is seen in the majority of cases and histological examination of the skeletal muscles of affected lambs soon after birth has shown no evidence of fibrosis. The cause could be a deficiency of vitamin E or selenium in the dam, an idiopathic primary ischaemia (perhaps due to a hereditary factor), or an ischaemia resulting from prolonged labour. Middleton (1934) stated that degeneration of the muscles and replacement by fat was observed in foetuses some weeks before full term. Miller (1960) states that in his opinion the muscle lesions in the Welsh Mountain lambs arose as a result of a primary ischaemia. Whatever the primary cause, it seems reasonable to assume that where the muscle lesions are slight in extent, repair and spontaneous recovery take place within a few weeks after birth, carriage of the head becomes normal and no permanent damage is caused. On the other hand, if the lesions in the neck muscles are severe and extensive, the defects can only be made good by fibrous tissue and there is subsequent contracture. The bilaterally symmetrical distribution and inelasticity of the fibrosis overlying C6, C7 and T1 prevent normal growth of these vertebrae, T1 apparently being most severely affected, and wedge-shaping is the result. The adult animal is then rendered quite unable by the con-

formation of these vertebrae to raise its head above the level of its back. At the moment no reason can be offered for the particular vulnerability of the longissimus cervicis muscle to damage, or for the consistent bilateral symmetry of the lesions, a well-recognised feature of myopathy due to deficiency of vitamin E or selenium.

An obvious gap in our knowledge of this condition is the conformation of the vertebrae in affected lambs at birth. However, since the great majority of these lambs quickly recover with no permanent deformity, there is considerable presumptive evidence that the vertebrae are not primarily affected.

CONCLUSIONS

Flexed limb joints and inability to raise the head in Suffolk lambs at birth were found to be due to a congenital myopathy, apparently originating shortly before birth. The majority of cases recovered spontaneously within a few weeks, but in a few instances fibrous contractures of neck muscles caused permanent deformity of cervical and thoracic vertebrae. The cause of the myopathy is not known.

ACKNOWLEDGMENTS

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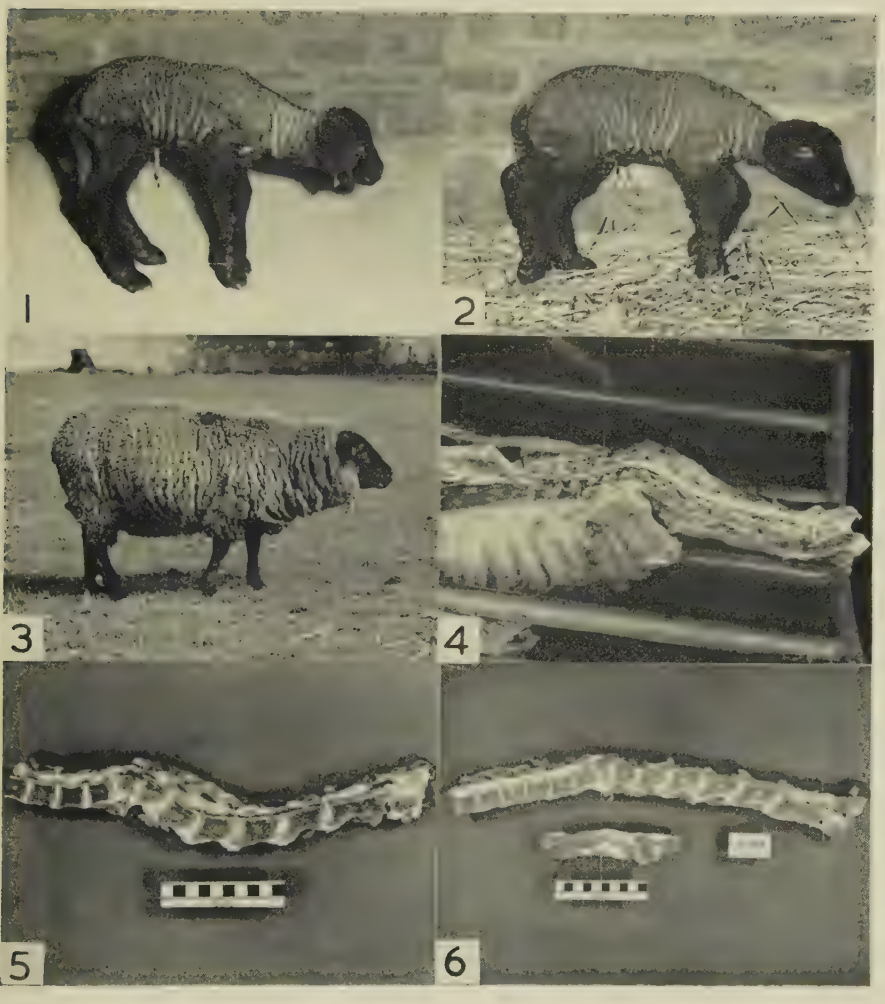

Fig. 1. Ewe lamb, within a few hours of birth, unable to raise its head.

Fig. 2. Ewe lamb, 6 days old, unable to raise its head.

Fig. 3. Ewe hogg aged 15 months, with abnormal head carriage. Elevation at cervical-thoracic junction can be seen.

Fig. 4. Hogg. Cervical and anterior thoracic region. Note elevation at cervical-thoracic junction.

Fig. 5. Hogg. Vertical section of cervical and thoracic vertebrae from C1 – T4. The wedge-shaping of C7 and T1 and their displacement dorsally are obvious.

Fig. 6. Wether, aged 3½ years. Vertical section from C1 – T7, showing wedge-shaping and dorsal displacement of T1. Below is one of the longissimus cervicis muscles showing fibrosis overlying T1.

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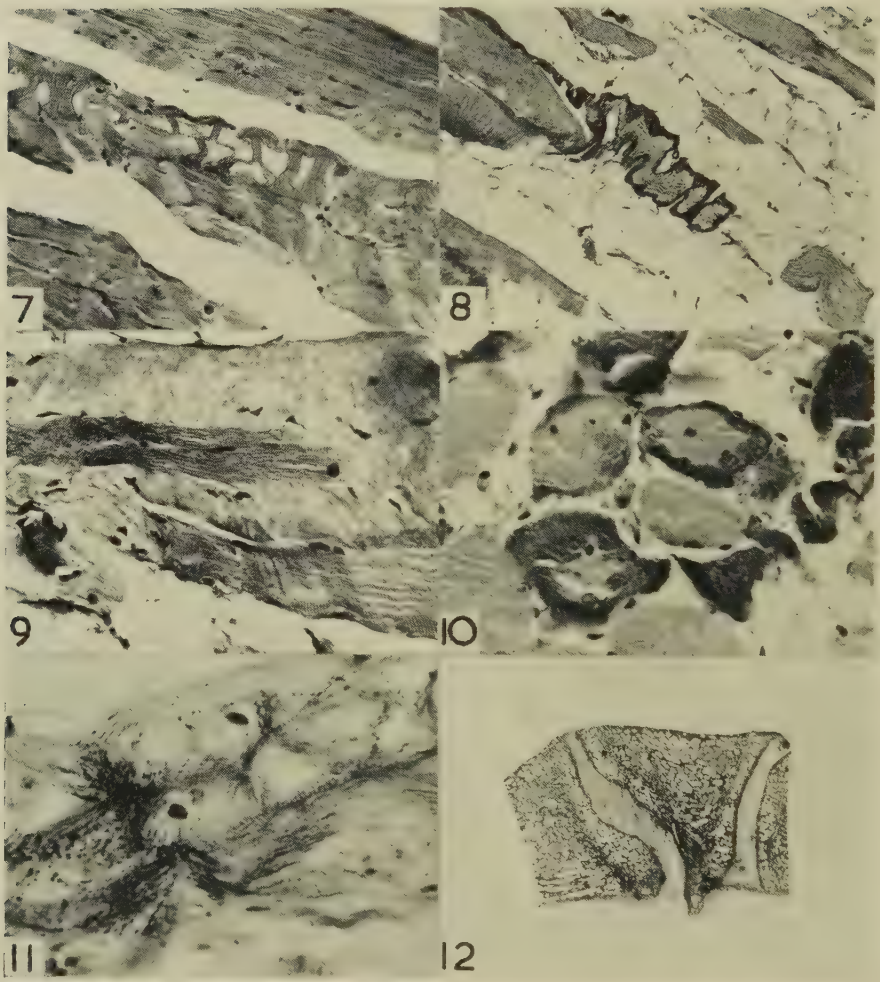

Fig. 7. Wether. Neck muscle showing necrosis and segmentation. H and E × 100.

Fig. 8. Wether. Neck muscle showing fat replacement and encapsulation of a muscle fibre by fibrous tissue. Van Gieson × 100.

Fig. 9. Wether. Neck muscle. A muscle fibre is seen with clearly defined striations, yet enveloped in fibrous tissue. H and E × 260.

Fig. 10. Hogg. Neck muscle. Transverse section, with fibrous tissue surrounding individual fibres. Van Gieson × 360.

Fig. 11. Hogg. Heart. Subendocardial Purkinje fibres showing degenerative changes. Phosphotungstic acid-haematoxylin × 410.

Fig. 12. Wether. Vertical section through vertebrae. From left to right C7, T1 and T2. The wedge-shape of T1 is obvious. There is no evidence of fracture. H and E × 4/7.

A MALIGNANT INTRAOCULAR MELANOTIC TUMOUR IN A GOAT

By

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INTRODUCTION

Cutaneous melanotic tumours are common in domestic animals, but the intraocular type is relatively rare. Saunders and Barron (1958) found less than 50 reported cases of intraocular tumours in animals and of these they classified 40 as "primary pigmented intraocular growth". These occurred in dogs, cats, horses, sheep, cattle, rabbits and chickens. Thomas (1929), in his study of melanotic skin cancer in South African goats, found no intraocular tumour, primary or metastatic, in a series of 24 animals, and as far as the present writers are aware, intraocular melanotic growth has not been reported in the goat.

The purpose of this paper is to report a case of malignant melanotic tumour in a 2-year-old female goat which we believe to be of intraocular origin.

METHODS

Tissues were fixed in 10 per cent formalin and stained with haematoxylin and eosin. Selected sections were stained with the Heidenhain method for connective tissue and the Wilder method for reticulum fibres.

RESULTS

Clinical History

When fourteen days old, the upper eyelid of the goat was completely destroyed by a caustic dehorning paste. Subsequently, exuberant granulation developed along the corneoscleral margin, and there was a severe conjunctivitis and keratitis, with vascularisation of the cornea. Complete ablation of the eye was performed at twenty months of age; no gross abnormality was observed at surgery.

Satisfactory union of the suture line did not occur and an area of granulation tissue approximately 0·5 cm in diameter remained in the centre of the orbit. The wound was then treated with a dusting powder containing copper sulphate and charcoal, but failed to heal. When performing débridement of the area, it was noticed that a thin line of black tissue approximately 3 to 4 mm in diameter was present in the orbit. The significance of this was not apparent, as the presence of the charcoal in the dusting powder obscured the picture, and the wound subsequently healed. Six months later, the animal was presented at the University of Queensland Veterinary School Clinic

with a history of rapid loss in condition, depression and anorexia. She was said to be three months pregnant and exhibited a distended abdomen. A Rothera's test on the urine was strongly positive for ketone bodies. Treatment with A.C.T.H. and intravenous dextrose for a week greatly improved the animal's demeanour and appetite. A week after discharge the goat became depressed, exhibiting complete anorexia and marked respiratory embarrassment. Death occurred one week later.

Post Mortem Findings

Macroscopic examination. The abdominal cavity contained about 3 litres of blood-stained fluid with strands and flocculi of black material. Nodular deposits of the black material were seen under the peritoneal lining of the abdominal wall, under the serous surfaces of the forestomachs and abomasum, and the bladder and its laterial ligaments, as well as in the aponeurosis of the abdominal muscles. A striking discolouration occurred in the omentum, caused by subserous deposits of the black material in irregular pattern, particularly in the depressions between the arboreous ridges of the omentum, and covering approximately a quarter of its total surface (Fig. 1).

The liver was grossly distorted and moderately enlarged; it was united to the diaphragm, abdominal wall and reticulum by fibrinous and fibrotic strands. The distortion and enlargement was caused by five fluctuating and coalescing tumours measuring from 7 to 15 cm in diameter and protruding on the visceral and diaphragmatic surfaces. These growths were thickly encapsulated and greyish in colour. When cut open, the central areas contained semi-fluid black material surrounded by nodulated growth of patchy pigmentation. The liver tissue was yellowish-orange and soft.

Several subserous nodules up to 5 mm in diameter were seen in the spleen. Otherwise the organ showed no change. The kidneys were slightly enlarged and softened. Several black nodules up to 6 mm in diameter were seen in the cortex of both adrenals, and under the serous lining of the uterus. The latter contained two 140 mm foetuses which showed no significant changes. The bladder was distended and contained black coloured urine.

The thoracic cavity contained about 30 ml. of straw-coloured fluid with a small amount of coagulated fibrin. The mucosa of the trachea and bronchi showed congestion, and petechial haemorrhages were seen in the latter. The lungs were congested and oedematous; several subserous black nodules of 12 cm in diameter were seen on all lobes. Groups of smaller lesions were found on the diaphragmatic lobe of the right lung. The pericardial sac contained about 20 ml. of slightly reddish fluid. Several black nodules measuring about 5 mm. in diameter were seen under the epicardium, which also showed ecchymoses. The myocardium was softer and more yellowish than normal. There was a considerable number of ecchymoses in the thymus.

The right orbit was covered by skin, and the surgical wound had healed. When the skin was incised, a black nodulated growth which nearly filled the orbital cavity, was seen (Fig. 2). The growth was firm and the cut surface showed patchy pigmentation, with a few necrotic foci. The medial border of the growth infiltrated the osseous wall of the orbit and abutted on to the turbinate bones.

The prescapular, maxillary and retropharyngeal lymph nodes were reddened and oedematous. Of the superficial lymph nodes of the body, only the right parotid and supramammary nodes showed pigmentation. The former also showed an increase in size with nodulation. The other superficial lymph nodes, namely the prescapulars, the superficial cervicals, the prefemorals and the popliteals were normal. The lymph nodes of the thoracic cavity, namely the intercostal, costocervical, anterior sternal, sternal, bronchiolar and the posterior mediastinal nodes showed intense pigmentation but without apparent increase in size. In contrast, the lymph nodes of the abdominal cavity, i.e. the portal, renal and internal illiac nodes were pigmented as well as enlarged. The sublumbar lymph nodes showed pigmentation, but were not increased in size.

An appreciable amount of oedema was present in the brisket and the ventral parts of the body.

Histological examination. The parenchyma of the tumour consisted of two main types of cells, epithelioid and spindle cells. The epithelioid cells contained oval or round pale nuclei with distinct nucleoli (Fig. 3); the cytoplasm was slightly acidophilic and scanty, so that most cells appeared as naked nuclei; their size varied, the larger ones measuring about 14·5 μ, the smaller ones about 3·5 μ in diameter. The spindle cells had elongated nuclei and more abundant cytoplasm. Fine dark brown granules of melanin were found in both types of cell. In some of the epithelioid cells they were small in number, while in others they were altogether absent. The spindle cells, however, were more heavily pigmented, and in many instances the cytoplasm was so packed with pigment granules that cellular details were obscured (Fig. 4). The interstitial stroma varied in the different sites; this will be mentioned later, together with the description of the various metastases. Apart from the tumours, a large amount of melanin was present in histiocytes and reticular cells, particularly in the serosa of the omentum, the peritoneum and in lymph nodes. These melanophages measured up to 30 μ in diameter and were filled with coarse pigment granules.

In the orbital tumour both types of tumour cell were seen. In some areas, there were small and large epithelioid cells, sometimes showing alveolar formation between scanty interstial stroma (Fig. 3). In other areas, the spindle cells were the major component; here, the interstitial stroma was more abundant and fasciculation was evident. There were a few necrotic areas with free melanin granules in the debris.

In the liver metastases, the cellular elements were mainly of the

epithelioid variety, with more prominent alveolar formation than in the orbital growth. Pigmentation was moderate in some areas, while in others it was totally absent. The capsules of these metastases, which consisted of compressed fibrotic liver tissue, were well developed, but in some areas tumour infiltration occurred. Large areas of necrosis were seen in the centres of these metastases, some showing perivascular zones of viable tumour cells with intervening necrosis—the so-called 'perithelial formation' of Harvey, Dawson and Innes (1940). The intervening liver tissues showed extensive fatty infiltration as well as haemorrhages. Bile pigments were abundant in the canaliculi. In some areas hepatic cells appeared as homogenous eosinophilic masses and only the Kupffer cells and cells of the reticulohistiocytic system remained visible.

Pulmonary metastases contained spindle cells mainly and showed considerable infiltrative growth. Many metastases were found in terminal bronchi and bronchioli, the smaller of these being within the bronchiolar space and showing little infiltration in the peribronchiolar tissue. The others showed less of the outline of the bronchiolar wall, and tumour cells infiltrated the surrounding tissues, obliterating the alveolar appearance of the normal lung. At the outer edges of these areas, pulmonary alveoli were filled with tumour cells as well as desquamated phagocytic cells containing pigment. Farther away from the metastases, pigment granules were seen in the histiocytic cells of the alveolar wall. Many of the small branches of the pulmonary artery contained thrombotic plugs of tumour cells. The pigment content of these pulmonary metastases varied from moderate to abundant, but there were no areas where the pigment was totally absent.

In the peritoneum and omentum metastatic foci were small. They were encapsulated, but contained very little interstitial stroma. The tumour cells were of epithelioid variety and contained a moderate amount of pigment. There were also numerous melanophages which often occurred in groups and could be mistaken for true metastases. However these cells were large and the pigment granules they contained were coarse.

The right parotid lymph node was wholly replaced by tumour tissue which varied in its cellular composition. The fascicular formation in some parts was more marked and the pigment more abundant than the corresponding areas in the orbital growth. There was an area where the cells were almost altogether free from pigment; these cells were of the epithelioid type. A large amount of pigment was seen in the bronchial node, but it was mainly within the cytoplasm of the reticular cells. There were also small foci where pigment-bearing cells aggregated: these appeared to be young metastases. In the costocervical node several small metastases were present. The cells were large and round, and the pigment was abundant. Melanophages were also seen in the node. The supramammary node showed extensive metastases, in which the cells were large and contained

much pigment. The normal structure of the node, however, was not altogether altered. There were no metastases or phagocytosed pigment in the prescapular, and the right and left retropharyngeal nodes.

In the spleen, the metastases were composed of moderately pigmented epithelioid cells. The serosa also contained numerous melanophages. The splenic tissue itself was unaffected. In the adrenals, metastases were confined to the cortical areas. The cells were of the epithelioid type with moderate pigment content. The interstitial stroma was scanty and alveolar formation was evident in some parts. A few 'perithelial formations' were also seen. No metastases were observed in the myocardium.

The reticulum content of the tumour, as revealed by Wilder's technique, varied from moderate to absent. In the orbital growth, only the areas showing fasciculation showed any substantial amount of reticulum fibres, while in the epithelioid areas the reticulum content was small (Fig. 5) and in some liver metastases it was altogether absent.

DISCUSSION

Melanotic tumours are either of cutaneous or ocular origin (Harvey *et al*, 1940; Wright, 1954). In the present case, there was no tumour in the skin, nor were the cutaneous regional lymph nodes involved, except for the right parotid and the supramammary nodes. These, however, are also regional for other areas, the parotid draining part of the orbital cavity and the supramammary the posterior part of the abdominal wall. As there was a tumour in the right orbit and pigment deposit in the aponeurosis of the abdominal muscles, it is justifiable to assume that the metastases in the two lymph nodes were from these non-cutaneous tributary areas. Despite the widespread metastases in the abdominal and thoracic cavity, only the right orbit and the right parotid lymph nodes were involved in the head and neck region. This distribution indicates that the tumour was of ocular origin and had already invaded the extraocular tissue at the time the eye was removed. From here it metastasized to the parotid lymph node and the other organs. It is strange, however, to find no changes in the superficial cervical and the retropharyngeal lymph nodes. In other ruminants it is known that communication occurs between these nodes and the parotid, but no information on the lymphatic system of the goat is available.

The ocular origin of the tumour is supported by the clinical history of a probable eye damage caused by the caustic dehorning paste, and the delay of healing after the surgical removal of the eye. Whether the paste and the subsequent irritation had actually caused the tumour cannot be verified as the eye after removal was not subjected to a pathological examination.

The tendency of ocular melanotic tumours to metastasize to the liver has been noted by Saunders and Barron (1958). In our case the

most extensive metastases were found in this organ. They consisted mainly of the epithelioid cell type, as did the other abdominal metastases. The lung metastases were associated mainly with blood vessels and bronchi in the form of thrombotic plugs. Those in the vessels were probably the result of haematogenous spread, while those in the bronchi were probably derived from tumour cells which had penetrated into the lumen and spread by progressive extension and implantation metastasis. The tumours were small and consisted mainly of spindle cells.

While we can thus say with reasonable certainty that the tumour is ocular, it is more difficult to determine its exact origin within the eye. Pigmented cells in the eye are firstly, the cuboidal epithelial cells of the retina, ciliary body and iris, and secondly, the dendritic cells of the sclera, choroid, ciliary body and iris. The spindle cells in the tumour under consideration indicate an origin from the dendritic cells. The possibility of the tumour having developed simultaneously from two different tissues is very remote, in spite of the considerable number of epithelioid cells present. On the other hand, it is known that primitive and relatively undifferentiated cells of any origin may assume an epithelioid appearance. A low degree of differentiation conforms with the high degree of malignancy exhibited in our case.

Wilder and Paul (1951) introduced diagnostic criteria for the determination of the degree of malignancy in ocular melanotic tumours in man, based on cell type and reticulum content. Six cell types are recognised, namely spindle A, spindle B, fascicular, necrotic, mixed and epithelioid, in order of increasing malignancy. The reticulum contents, determined by Wilder's reticulum stain, may be heavy, medium, light or absent, in order of increasing malignancy. Saunders and Barron (1958) indicated that the criteria were valid in dogs. In our case, though the metastases were predominantly epithelioid, the tumour was of the mixed type, especially in the primary growth. The reticulum content in the fasciculated areas in the primary growth was moderate, but in the epithelioid regions and in the metastases, it was light and even altogether absent. Thus, Wilder's histological criteria place the tumour under discussion among those of a very high order of malignancy, in conformity with the clinical as well as the macroscopic findings.

The classification of melanotic tumours generally is still largely unsettled because of the uncertainty regarding the origin of the melanoblasts. It was believed that epidermal melanoblasts were mesodermal and on this basis malignant melanotic tumours were classified as sarcomas. Since Borcea (1909) suggested that melanoblasts in fishes originated from the neural crests, an increasing amount of evidence has been gathered showing that this was also true in the higher vertebrates (Harrison, 1910; Weidenreich, 1912; Du Shane, 1943, 1944; Dorris, 1936, 1939; Eastlick, 1939; Rawles, 1940; Masson, 1926). Although Goldberg (1948) maintained that some melanoblasts at least are mesodermal, there is a general

tendency now to accept the neuroectodermal theory. On this basis, malignant melanotic tumours are regarded as carcinomas and Harvey *et al.* (1940) excluded altogether the possibility of melanosarcomas.

It is generally accepted that in the eye, the pigmented epithelial cells of the retina, iris and ciliary body are of neuroectodermal origin, whereas the dendritic pigment cells in the iris, ciliary body and choroid are held by some to be mesenchymal (Cappell, 1958). However, the matter is still unsettled.

To summarise the situation, perhaps the words of Willis (1948) are pertinent: "Much of the confusion regarding the histogenesis of such tumours has arisen from supposing them all to be of similar origin—all mesodermal, all epithelial, or all neuroectodermal. Let us . . . abandon this assumption and consider separately the several groups of melanotic tumours according to their site of origin." It is with this view in mind that we choose the term malignant intraocular melanotic tumour.

The ketosis seen clinically in our case can be explained by the extensive liver damage. The abdominal distention was the result of the hydroperitoneum and, to a lesser extent, the hepatomegaly. Together they caused an increased intraperitoneal pressure, which explained the respiratory embarrassment seen ante-mortem. Urine discolouration was probably due to excreted melanin, a condition usually seen in cases of malignant melanotic tumour with extensive necrosis.

Another point of interest in this case was the age of the animal. Little is known about the relationship between age and tumour incidence in the goat, but melanotic tumours, at least the cutaneous type, are apparently uncommon in animals younger than 3 to 4 years old. In the series studied by Thomas (1929), it occurred in only one animal younger than "four-tooth". However, three cases of pigmented iridal tumours occurring in young dogs (8, 9, and 12 months old) have been reported (Saunders and Barron, 1958; Bloom, 1942; and Buckler, 1933).

CONCLUSIONS

A malignant melanotic tumour in a 2-year-old female goat, showing polyvisceral metastases, is described and a description of the macroscopic and histological findings is presented. The evidence available indicates that the tumour originated intraocularly, an event which has not previously been reported in the goat.

The application of Wilder's criteria for malignancy in melanotic tumours supported the other evidence indicating that the tumour was highly malignant.

The controversy surrounding the origin of the melanoblasts and the classification of melanotic tumours is briefly discussed.

ACKNOWLEDGMENT

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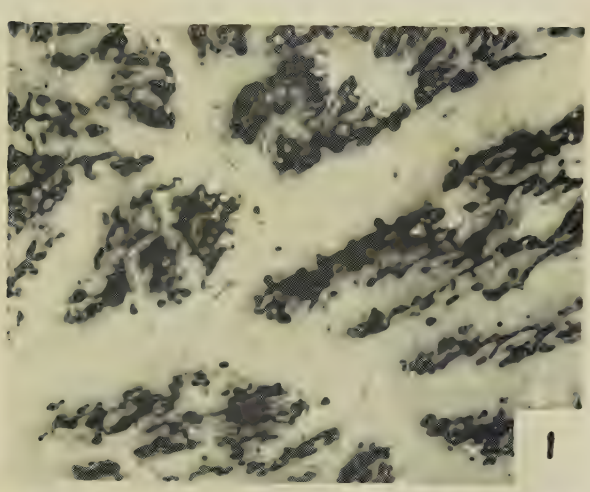

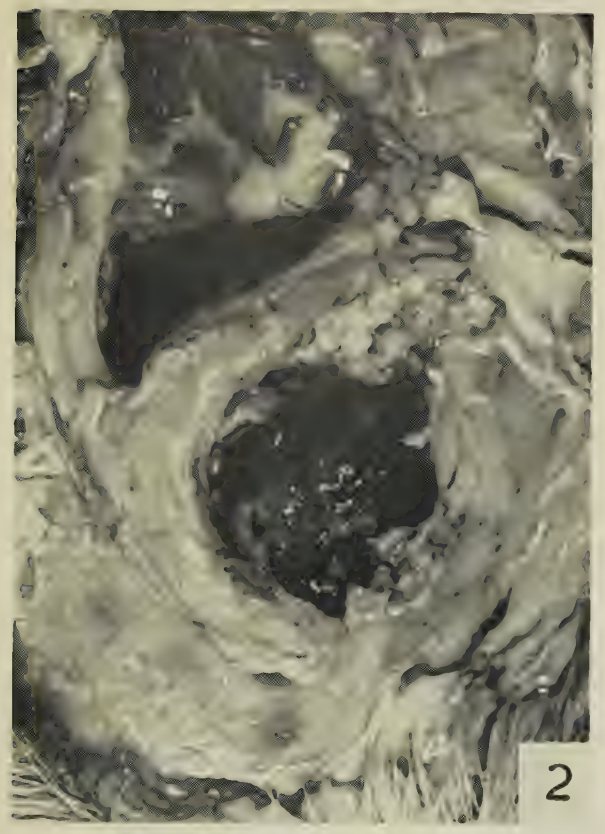

Fig. 1. Irregular subserous deposit of melanin in omentum, particularly in depressions between its arboreal ridges.

Fig. 2. Nodulated growth in the right orbit.

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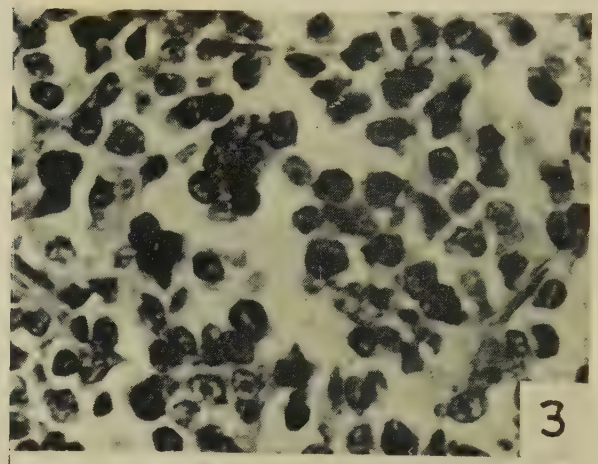

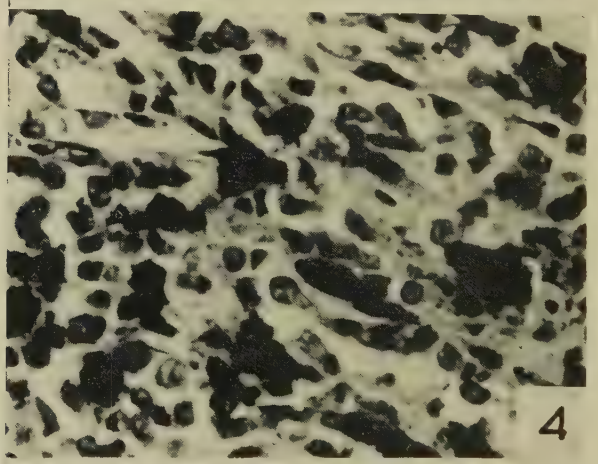

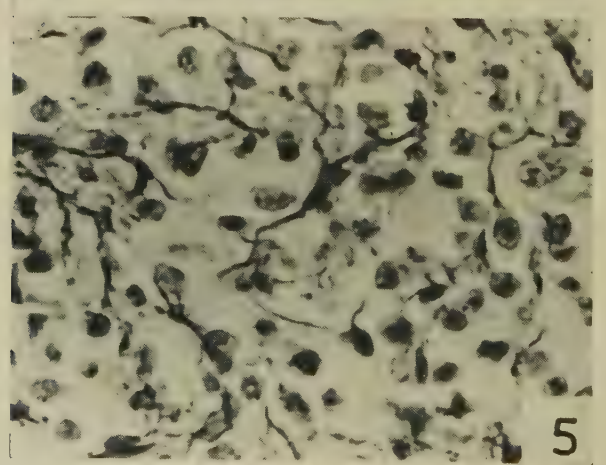

Fig. 3. Poorly pigmented area in orbital growth, where epithelioid cells predominate. (H & E, 600 ×).

Fig. 4. Heavily pigmented area in orbital growth showing numerous spindle cells containing melanin granules. (H & E, 600 ×).

Fig. 5. Light reticulum content of an epithelioid area of the orbital growth. (Wilder's reticulum stain, 600 ×).

OBSERVATIONS ON CALVES VACCINATED WITH IRRADIATED LARVAE OF *DICTYOCAULUS VIVIPARUS*

Serum antibody Titre, Respiratory Rate and Response to Challenge

By

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INTRODUCTION

The use of irradiated larvae of *D. viviparus* in the production of immunity to bovine parasitic bronchitis has been described by Jarrett, Jennings, Martin, McIntyre, Mulligan, Sharp and Urquhart (1958); Jarrett, Jennings, McIntyre, Mulligan, Sharp and Urquhart (1959) and Jarrett, Jennings, McIntyre, Mulligan and Urquhart (1960).

From their early observations they concluded that irradiated larvae underwent a limited migration as far as the mesenteric lymph glands, where they died after producing their immunogenic effect (Jarrett, Jennings, McIntyre and Mulligan 1957). It was found, however, that a rise in respiratory rate occurred in the majority of calves 15 to 20 days after the first dose of vaccine, and this indication that larvae were developing in the lungs was confirmed by the finding of lesions post mortem (Jarrett *et al.* 1959) and a small number of adult worms (Jarrett *et al.* 1960).

Cornwell (1960b) suggested that the irradiated larvae developing in the lungs were responsible for the stimulation of immunity, and that the differing degree to which the larvae became established in the lungs was reflected in the variable complement fixing (C.F.) antibody response and degree of immunity. Poynter, Jones, Nelson, Peacock, Robinson, Silverman and Terry (1960) clarified the position when they found that irradiated larvae migrated to the lungs in the same way as normal larvae, but did not as a rule develop to the adult stage. They also observed that in the resistant calf normal larvae migrated directly to the lungs where they are subjected to the immune reaction of the host. No evidence was found that the larvae were held up during their passage through the mesenteric lymph nodes. These workers did not find that the mean respiratory rate of 12 calves given a single dose of 1,000 irradiated larvae rose beyond normal limits.

In their experiments with calves given a double dose of vaccine and subsequently challenged with 10,000 larvae, Jarrett *et al.* (1959) found the calves to be completely resistant, with no increase in respiratory rate and no larvae in the faeces after the challenge dose. Cornwell (1960b), however, did not find calves similarly vaccinated to be completely resistant, either to the effects of a field challenge or to challenge with 10,000 larvae.

Serological studies using the C.F. test and a heated whole worm antigen revealed little or no response to a single dose of vaccine but a rapid anamnestic response to challenge (Jarrett *et al.* 1960). Cornwell (1960a) also found a negligible response to the first dose of vaccine, and although the second dose did usually "boost" the titre, great variation in

response occurred in individual calves. Subsequent challenge stimulated an early increase in titre in almost all calves (Cornwell, 1960b).

The present paper records observations, made on four farms, on the serum antibody titre and respiratory rate in 26 calves vaccinated with irradiated larval vaccine. Four of the calves were later challenged at the laboratory.

METHODS

On four farms 26 calves varying in age from 2 to 6 months and reared artificially in loose boxes under conditions of good farm hygiene were vaccinated with a double dose of irradiated larval vaccine* at a dosage interval of 4 weeks. Respiratory rates were recorded, and faeces and blood samples taken. Seven unvaccinated calves on two of the farms served as controls for the observations on respiratory rate.

Ten weeks after the second dose of vaccine, the 4 vaccinated calves from farm A were challenged indoors at the laboratory with 5-10,000 larvae, according to size. Two unvaccinated laboratory calves housed under the same conditions served as controls for the respiratory rate.

The calves on the other farms were turned out to graze 6 weeks after the second dose of vaccine. Table 1. gives a summary of the distribution of the calves on the various farms.

C.F. tests using heated whole worm antigen were carried out as previously described (Cornwell and Michel, 1960) and faeces examinations were performed on 10 g. of rectal faeces by the Baermann method.

RESULTS

Farm A. On this farm 4 vaccinates and 2 controls were observed (Fig. 1). The vaccinates showed a slight increase in respiratory rate compared with the controls during the 4 weeks between the two doses of vaccine, the mean response reaching 42 per. min. at the 22nd day. After removal to the laboratory and challenge, the respiratory rate increased from its pre-challenge level of 38 to a peak

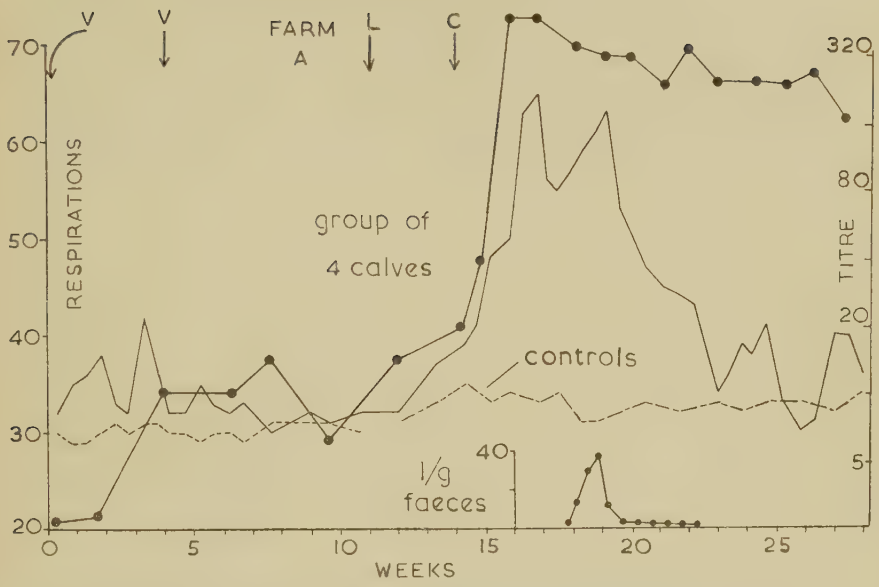
TABLE 1

DISTRIBUTION OF CALVES BETWEEN THE FARMS

Farm	*Number of vaccinates*	*Number of controls*	*Treatment after vaccination*	
A	4	2	Vaccinates removed to laboratory and challenged with 5-10,000 larvae.	Two laboratory calves acted as controls for respiratory rate.
D	6	0	Went out to grass	
H	4	0	Went out to grass	
P	12	5	Went out to grass	
Total	26	7		

*"Dictol"—Allen and Hanburys Ltd.

Fig. 1.



Mean respiratory rate, C.F. antibody titre and faecal larval counts of 4 calves vaccinated on farm A and later removed to the laboratory for challenge.
●—● =antibody titre. V=dose of vaccine. C=challenge.
——— =respiratory rate of vaccinates. L=removed to laboratory.
– – – – =mean respiratory rate of 2 unvaccinated calves on farm A.
– – – – =mean respiratory rate of 2 unvaccinated calves at laboratory.

of 65 at 2 to 3 weeks and then declined to the normal range (25 to 40) after 8 to 10 weeks. The 2 laboratory control calves had respiratory rates well within the normal range. Clinical symptoms were present after challenge in these calves but no setback was apparent. Larvae appeared in the faeces of all 4 calves 25 days after challenge, the highest count being 70 larvae/g. in the calf which received 5,000 larvae. The period of larval excretion was 10 to 31 days.

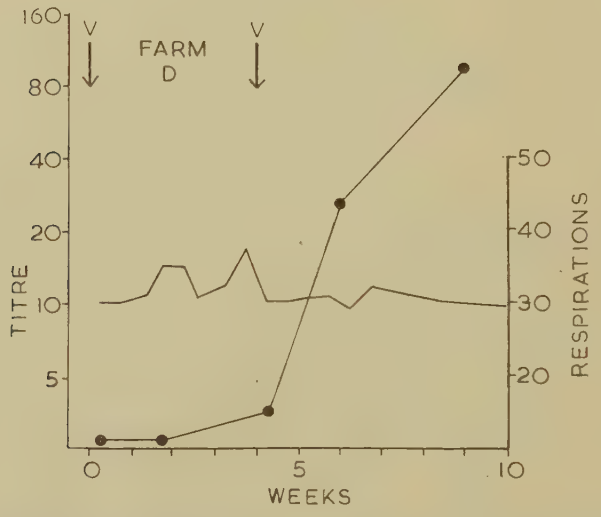
The individual C.F. antibody titres varied from 0 to 10 at ten weeks (Fig. 5). The mean titre was 10 at the time of the second dose of vaccine and 20 at challenge. A rapid secondary response followed the challenge, reaching a peak of 450 in two weeks, followed by a gradual decline to 160 eleven weeks later (Fig. 1).

Farm D. The mean respiratory rate of these 6 calves remained within the normal range, the vaccination procedure having little effect (Fig. 2). In 2 calves the respiratory rate was slightly in excess of 40 per. min. 16 days after the first dose of vaccine.

The C.F. antibody titre had begun to develop in 3 calves at the time of the second dose of vaccine. Thereafter antibodies were stimulated in all 6 calves to a variable degree, the titres ranging from 5 to 480 at five weeks after vaccination (Fig. 5).

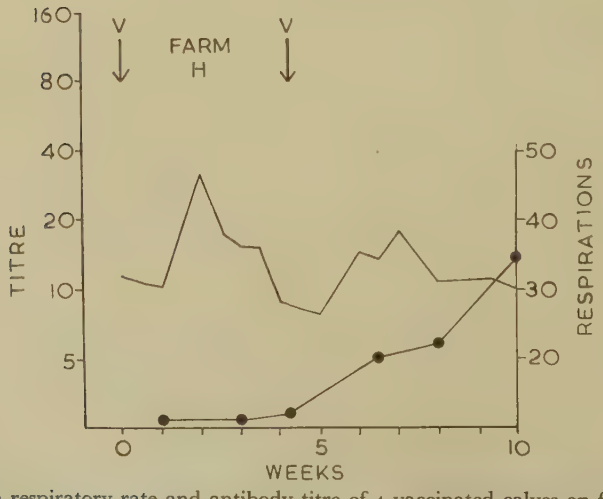
Farm H. The 4 calves showed an increase in mean respiratory rate between the two doses of vaccine, with a peak of 47 at 14 days (Fig. 3). The rise after the second dose of vaccine was within the normal range.

Fig. 2.



Mean respiratory rate and antibody titre of 6 vaccinated calves on farm D.
●—● = antibody titre. ——— = respiratory rate. V = dose of vaccine given.

Fig. 3.

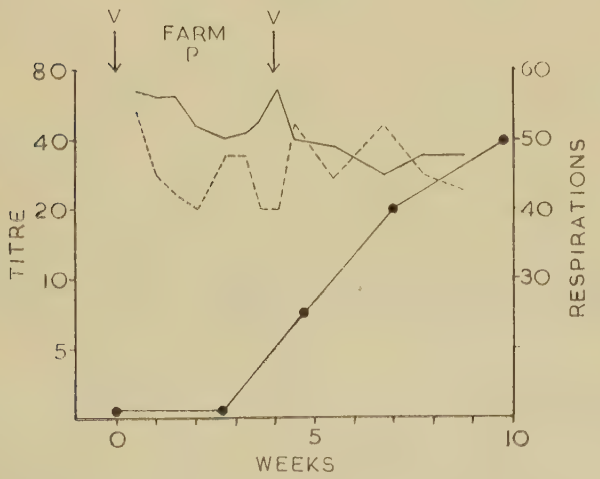


Mean respiratory rate and antibody titre of 4 vaccinated calves on farm H.
●—● = antibody titre. ——— = respiratory rate. V = dose of vaccine given.

Antibody titres after the second dose of vaccine had reached only low levels in 6 weeks and varied from 0 to 40 (Fig. 5).

Farm P. The circumstances on this farm differed from the other 3 farms in that a mild degree of respiratory disease ("virus pneumonia") was present amongst all the calves indoors, giving rise to coughing and an increase in respiratory rate. The 12 vaccinates and 5 controls showed respiratory rates higher than normal but no appreciable effect on the already elevated respirations resulted from vaccination (Fig. 4).

Fig. 4.

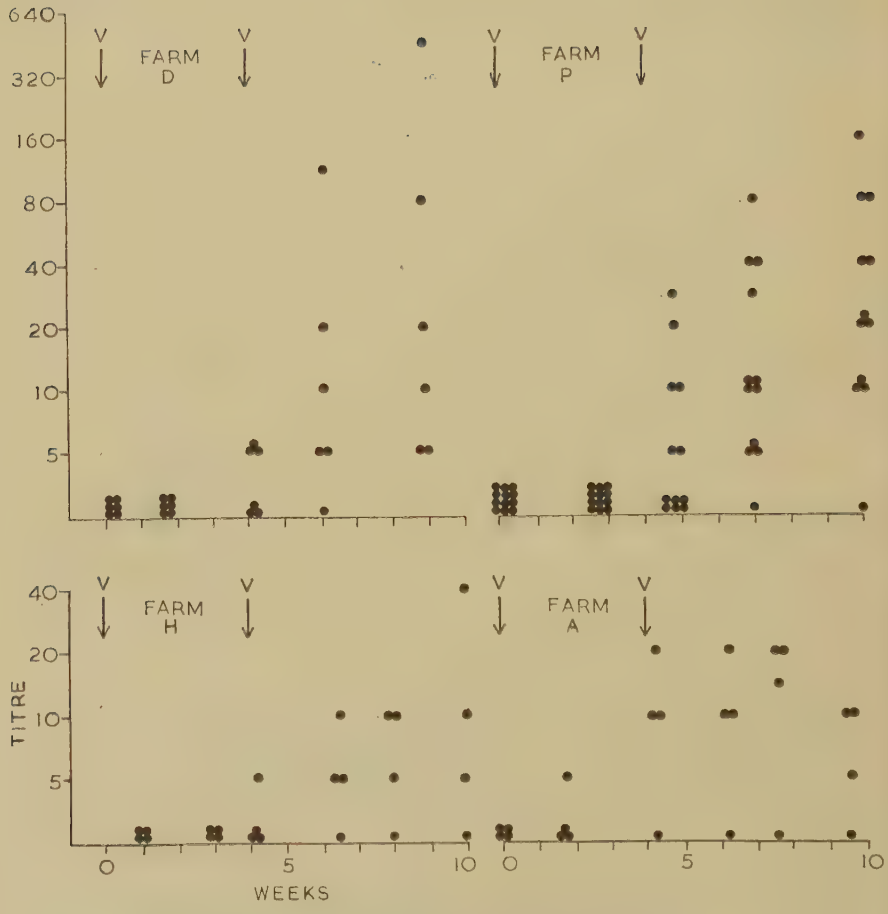


Mean respiratory rate and antibody titre of 12 vaccinated calves and mean respiratory rate of 5 control calves on farm P.
●—● = antibody titre. ——— = respiratory rate of vaccinates.
V = dose of vaccine given. – – – – = respiratory rate of controls.

All except one calf developed antibodies after the second dose of vaccine, the titre in individual calves varying from 0 to 160 (Fig. 5).

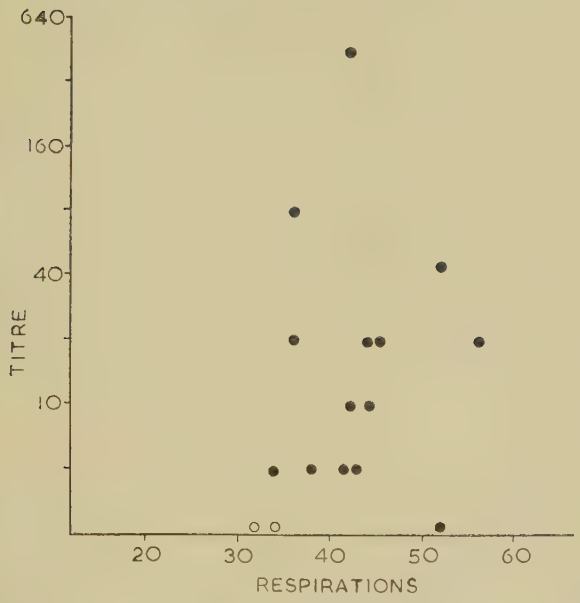
The calves on this farm were pastured some distance away from the buildings with a large group of unvaccinated yearlings. Faeces samples taken from the ground revealed this pasture to be infected in July, but it was not until September that the calves could be handled and sampled individually. At this single sampling 3 of the 12 vaccinates and 4 of the 5 controls were excreting small numbers of larvae. No clinical symptoms were reported from this group.

Fig. 5.



Individual antibody titres of 26 vaccinated calves on 4 farms.
V = dose of vaccine given.

Fig. 6.



Comparison between the highest recorded respiratory rate after the first dose of vaccine and the antibody titre 5-6 weeks after the second dose of vaccine in 14 calves. Two control calves are included for comparison (o).

DISCUSSION

Previous work (Cornwell, 1960a), showed that in calves given two doses of vaccine at intervals of 6 or 11 weeks between doses the C.F. antibody response was low after the first dose, and that although the second dose "boosted" the titre the individual calves varied widely in their response. In a group of calves vaccinated at an interval of 4 weeks between doses an unusually early onset of antibody response was noticed (2 weeks after the first dose) and it was suggested that this group might have experienced some sensitizing factor prior to vaccination. The present results with 26 calves confirm that this was probably so, as all these calves vaccinated at a 4 week interval reacted as did the previous ones vaccinated at longer intervals.

It was thought that the variation in antibody response shown by individual calves might reflect the extent to which the irradiated larvae were becoming established in the lungs. If the increase in respiratory rate is also an indication of the development of worms in the lungs, there might be some correlation between the C.F. titre and the rise in respiratory rate. In 10 of the 14 calves whose vaccination procedure was uncomplicated by existing respiratory disease, the respiratory rate was in excess of 40 between 14 and 22 days after

the first dose of vaccine and in 9 of these calves antibodies were later detected. Nevertheless, the remaining 4 calves whose respiratory rate showed no significant increase also developed antibodies. A comparison of the highest respiratory rate recorded within the period 14 to 22 days after the first dose of vaccine with the antibody titre developed at 9 to 10 weeks is shown in Fig. 6. It appears that although there is a general trend towards an increase in respiratory rate after vaccination and the development of a titre, there is no proportional relationship between these two values.

A consideration of the groups of calves on farms A, D and H reveals a slight increase in mean respiratory rate in two of them, with peak levels of 42 and 47 per minute—only slightly above the normal range. Jarrett *et al.* (1959), using 4 groups of 10 calves, found the peak response after the first dose of vaccine to vary from about 43 to 63 per minute. Poynter *et al.* (1960) on the other hand found the mean respiratory rate of a group of 12 vaccinated calves remained within the normal range. They also reported that irradiated larvae regularly migrated to the lungs. It would appear that present knowledge is insufficient to explain the great variation in the rise in respiratory rate and C.F. titre following vaccination.

The finding in previous work (Cornwell, 1960b) that vaccinated calves exposed to challenge may develop patent infections of low degree and a rise in respiratory rate is confirmed by the observations on the effect of challenge on the 4 calves from farm A. These calves resisted the challenge, but all of them developed light patent infections, a respiratory rate twice the normal and a rapid secondary response in C.F. titre (Fig. 1).

It is probable that several different factors contribute to the development of immunity to *D. viviparus* infection (Michel, 1957). Parker and Roberts (1958) found that calves infected with *D. viviparus* responded to treatment with diethylcarbamazine 14 to 18 days later by the suppression of the patent stage. The fact that these calves were slightly less resistant to challenge than untreated controls which had experienced a fully patent infection, led them to suggest that the adult worms may contribute to the development of immunity, as occurs naturally in the field. Michel and Cornwell (1959) reported that calves may develop a substantial resistance to reinfection as early as 12 days after an initial dose of larvae i.e. before adult worms are present. It would seem, therefore, that if the adult worms do contribute to the immune status of the host, their contribution is probably a small one. This contribution would be lacking in the majority of calves vaccinated with irradiated larvae, since the larvae do not usually mature in the lungs; it would be available to those vaccinated animals which acquire a low grade patent infection when turned out onto infected pasture. Such low grade infections are, in our experience, common among vaccinated calves exposed to natural or experimental challenge, thereby supplementing the vaccinal immunity.

In the present studies the administration of irradiated larvae to calves already suffering from "virus pneumonia" did not exacerbate the condition. The respiratory rate showed no increase over the existing high level, and the serological response followed the usual pattern. Whether the concomitant respiratory disease interfered with the development of immunity is not known because the severity of the natural challenge encountered later in the year could not be determined. Although clinical symptoms did not appear in this group, larvae were detected in the faeces of both vaccinated and unvaccinated calves grazing the same pasture.

No larvae were detected in the faeces of any calf prior to challenge, indicating the failure of irradiated larvae to produce patent injections. That this can occur, however, is shown by the work of Poynter *et al.* (1960) and Walley (1960), but its significance is probably small, and no evidence that the vaccine itself has produced an outbreak of disease has been reported.

CONCLUSIONS

Observations were made on 4 farms on the serum antibody titre and respiratory rate in 26 calves vaccinated at a four-week interval with two doses of irradiated *D. viviparus* larvae.

Ten weeks after the first dose of vaccine the serum titre to the C.F. test, using heated whole worm antigen, showed wide variations in individual calves ranging from 0 (three animals) to 480 (one animal).

In 10 of 14 calves on 3 of the farms the respiratory rate rose to just over 40 per min. 14 to 22 days after the first dose of vaccine, but the extent of this rise in individual calves was not proportional to the rise in the serum titre.

Four vaccinated calves removed to the laboratory and challenged with 5-10,000 larvae developed patent infections with the excretion of small numbers of larvae and a rise in respiratory rate to twice the normal value. The antibody titre showed a rapid secondary response.

On the fourth farm, where the respiratory rates were already raised by an infectious non-parasitic respiratory disease ("virus pneumonia"), vaccination caused no further rise in respiratory rate or other ill effect.

No larvae were detected in the faeces of any of the 26 calves prior to challenge or turning out to pasture.

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URINARY CALCULI IN THE DOG

I. Incidence and Chemical Composition

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INTRODUCTION

The incidence of urinary calculi in dogs, based on a sufficiently large number of cases, is difficult to obtain in an unbiased form because sampling of clinical cases is selective, and sampling of cases to be sent for chemical analysis is often even more so. Provided that sufficiently large numbers of cases are studied, however, such data on the incidence and composition of stones can provide useful information.

Klarenbeek, Langner and Raabe (1935) published data collected at the Utrecht small animal clinic covering 51 cases of urolithiasis. More comprehensive information was presented by White (1944, 1945), based on a study of 103 cases, mainly from the London area.

Figures from the Royal Veterinary and Agricultural College in Copenhagen, collected over a period of 15 years, were given by Krabbe (1949), and Brodey (1955) presented an analysis of 52 cases from the University of Pennsylvania over the period 1951-1954. A statistical survey of the incidence of canine urolithiasis in Stockholm was published by Krook and Arwedsson (1956). Finally, Uller (1959) has recently given the results of a survey of 554 cases seen at the clinic of the Veterinary High School in Vienna between 1928 and 1958.

The purpose of the present paper is to present data and conclusions on the incidence and composition of calculi based on a series of 122 cases collected between 1957 and 1960 by the writers and on a survey of calculus cases encountered at the Beaumont Animals' Hospital of the Royal Veterinary College, London over the years 1954-1959, viewing them against the background of dogs presented for all disease conditions over the same period. As it is probable that the incidence of calculi varies from one country to another, and since no figures have been published in Great Britain since 1945, the present survey gives an indication of the overall and relative incidence of calculi in different breeds in this country at the present time. Significant changes in the breed structure of the dog population and in the feeding of dogs have occurred since 1945.

MATERIAL AND METHODS

Sources of Calculi

The data on chemical composition is based on 122 specimens received from veterinary hospitals and practitioners in England, Scotland and Wales over the period 1957-1960. There has almost certainly been some selection by senders of the specimens in that not all of them have submitted every calculus they came across.

Information relating to breed, age and sex incidence has been obtained by examining the records of the Beaumont Animals' Hospital for 1954-1959, during which time 223 cases of urolithiasis were encountered. The ideal figures against which to view the cases of urolithiasis would be those for the dog population of the area from which the cases came. This is not obtainable and so we have used instead a sample of the dogs admitted to the hospital for all complaints over the same period. This sample consisted of all new cases presented at the clinic on the first and third Saturdays of each month throughout the six year period.

Methods of Analysis

Appearance. The gross features of calculi and their appearance when cut or broken often indicate their main constituents. Phosphate stones are usually white and are relatively loosely aggregated, at least in their central part. They range in size from fine gravel to stones larger than a hen's egg, and in number from single stones, which usually have a rough surface, to several hundred smooth, facetted, tetrahedral ones. Phosphate calculi yield a chalk-like powder when crushed. Urate stones are usually small and spherical; they are brittle and show concentric eggshell-like laminations. Cystine stones are creamy-yellow and smooth; they are usually small and spherical and can be crushed easily to produce a soft whitish powder. Oxalate stones are very hard and brittle and often show projecting crystalline shelves with very sharp edges which produce severe haemorrhage in the urinary tract. The features of the different types of stone which White described in 1944 were found again in the present series and no new types of calculus have come to light since then.

Routine microchemical analysis. A systematic method of qualitative microanalysis devised to use the minimum of material and easily completed within an hour was employed. It was more precise than the crude method described in 1944 in that it detected more readily the minor constituents.

The first step in the analysis for inorganic components is to heat the powdered sample of stone on a piece of porcelain in the oxidising flame of the bunsen. Organic matter which may interfere with systematic precipitation methods is burned away to carbon. Oxalate decomposes, first to carbonate and then to oxide: interference in the analysis for cations is now limited to phosphate. Cystine is immediately recognisable by the sulphurous odour.

The nature of the residue after heating often indicates the main constituent of the stone. A white residue which is alkaline indicates carbonate or oxalate; this must be confirmed with the original material. A white residue which is not alkaline indicates calcium phosphate, while a grey fused residue is typical of triple phosphate ($MgNH_4PO_4$, $6H_2O$). The effect of ignition on triple phosphate is to form magnesium pyrophosphate which appears as a grey, coke-like mass. A spot test with the fused sample, using ammonium molybdate, demonstrates the presence of phosphate.

The ignited residue is taken up into 0.5 N.HCl and the solution is freed from carbon by centrifuging. If phosphate is present the solution is treated with zirconium nitrate until no further precipitation occurs, when the zirconium phosphate is spun down. The supernatant is removed and made alkaline with ammonia to precipitate excess of zirconium nitrate. The supernatant after centrifuging is used to separate calcium and

magnesium. The pH is adjusted to neutral by aerating off the excess ammonia, and ammonium oxalate is added. A white flocculent precipitate of calcium oxalate may result and this is digested at 100°C until it becomes granular and settles. The precipitate is centrifuged and is confirmed as oxalate by its insolubility in acetic acid. The supernatant is evaporated to dryness, treated with concentrated nitric acid and slowly evaporated until white fumes cease to be evolved. This removes ammonium salts which interfere with the final spot tests for Mg and Na. The residue is taken up in a few drops of water. A blue precipitate with Magneson II reagent indicates the presence of Mg, and a yellow precipitate with zinc uranyl acetate reagent indicates Na. If a white or blue-white precipitate is obtained in the test for Mg it indicates that Ca or zirconium is still present and steps must be taken to remove them.

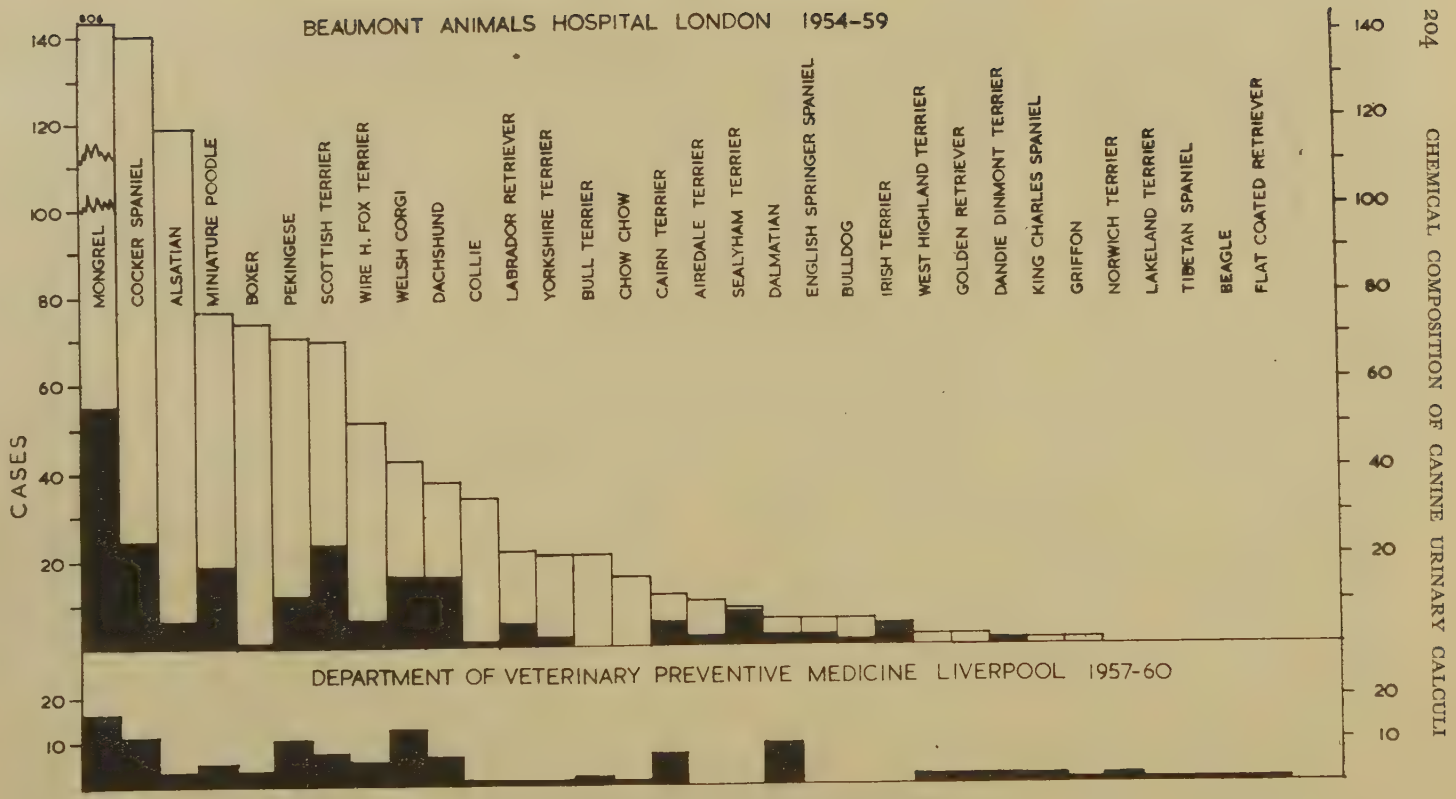
Further confirmatory tests are now performed on the original powdered sample of stone. The evolution of ammonia on warming with dilute NaOH indicates the presence of the ammonium ion, usually in the form of triple phosphate or ammonium urate. Carbonate is confirmed by the evolution of carbon dioxide when the powdered stone is treated with dilute acid. Oxalate is confirmed by the resorcinol test. We have confirmed the presence of silicate and sulphate in a calculus from a horse by testing a neutralised aqueous extract of a sodium carbonate digest of the powdered stone: silicate was shown by a white precipitate with hexammine zinchydroxide, and sulphate by a white precipitate with barium chloride and HCl. Specific tests for organic constituents are the nitroprusside test for cystine and the murexide test for urates.

Paper chromatography. A two-dimensional technique for amino-acids is used, with phenol/ammonia and 2:6 lutidine/water as solvents (Dent, 1948). Treatment with ninhydrin results in the highly specific identification of cystine (as cysteic acid) and other amino-acids. Elution of the spots makes a quantitative determination possible. Single dimensional chromatography for purines can be carried out using the system diethylene glycol/butanol/water (4:1:1) recommended by Vischer and Chargaff (1948). After development, treatment with mercuric acetate in 95 per cent ethanol, followed by spraying with diphenyl carbazone in 95 per cent ethanol and warming at 110°C make the purines visible as blue spots (Dikstein, Bergman and Chaimowitz, 1956).

Absorption spectra. A quantitative estimation of uric acid or urates can be carried out by using the characteristic ultraviolet absorption spectrum (λmax. 293 mμ). The determination is made on the sample dissolved in glycine buffer in a quartz cell of 10 mm. light path by following the change in extinction at this wavelength when the enzyme uricase is added. Urates also have a characteristic infra-red spectrum which enables a specific qualitative determination to be made. Ammonium urate is characterized by an infra-red spectrum which indicates hydrogen bonding, with a wide absorption peak at 3.4 μ. Most of the urate calculi so far examined have shown a very pure spectrum indicative of ammonium urate.

Optical and X-ray crystallography. Analysis of calculi by X-ray diffraction and by the use of the polarizing microscope on thin sections or powdered stone is a valuable supplement to microchemical analysis. A few preliminary studies with these techniques have confirmed the results of chemical analysis and have identified the specific crystalline constituents.

Fig. 1.



BREED INCIDENCE OF CANINE UROLITHIASIS

■ Total of calculus cases presented (Beaumont Animals' Hospital, 1954-59) and calculi received (Preventive Medicine, Liverpool, 1957-60).
□ Total of new canine cases (1,700) presented for all complaints at Beaumont Animals' Hospital on first and third Saturdays of each month in 1954-59.

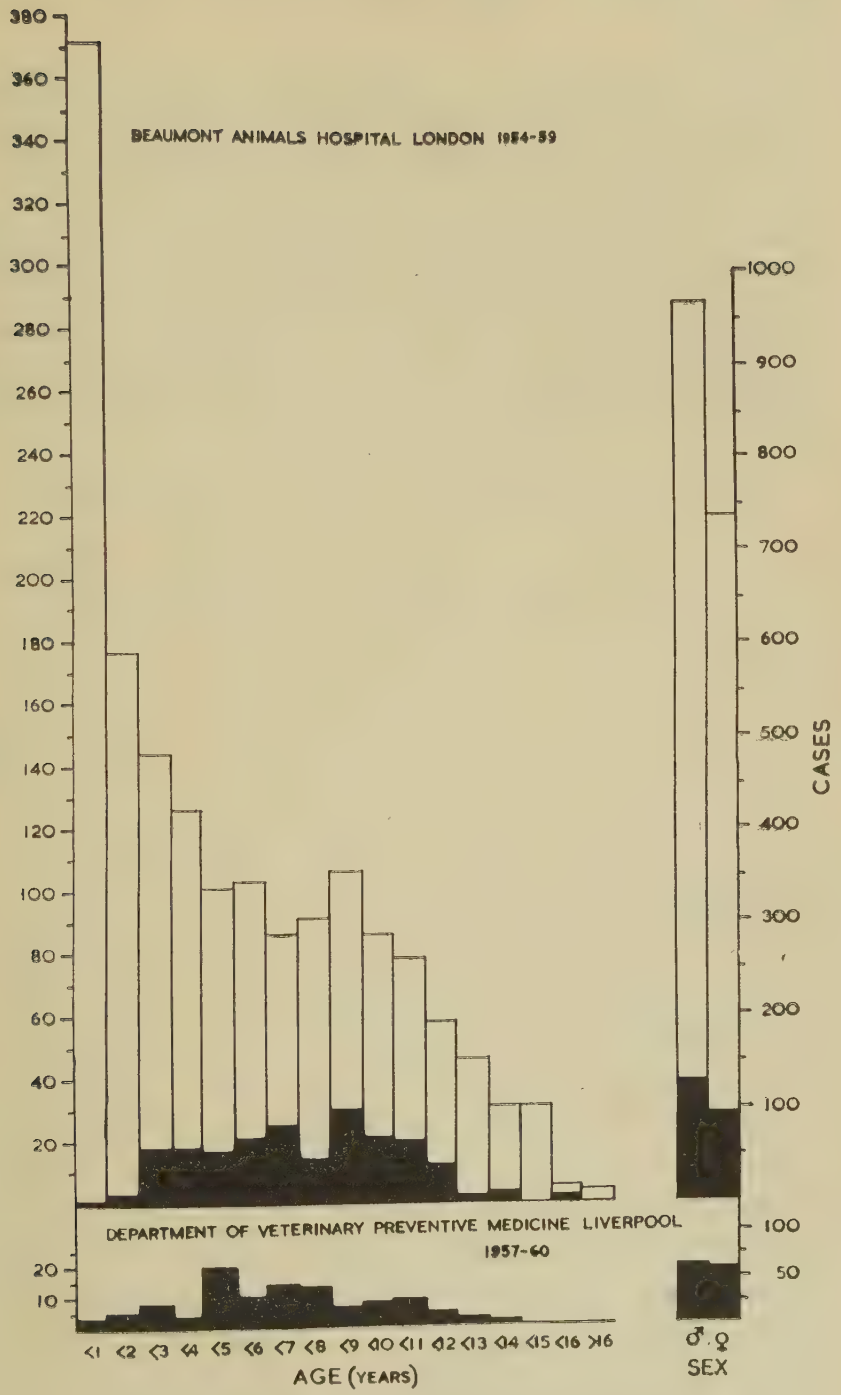

BEAUMONT ANIMALS HOSPITAL LONDON 1954-59
DEPARTMENT OF VETERINARY PREVENTIVE MEDICINE LIVERPOOL
1957-60
CASES
AGE (YEARS)
SEX
♂ ♀
<1 <2 <3 <4 <5 <6 <7 <8 <9 <10 <11 <12 <13 <14 <15 <16 >16

Fig. 2.

AGE AND SEX INCIDENCE OF CANINE UROLITHIASIS.

■ Total of calculus cases (Beaumont Animals' Hospital, 1954-59) and calculi received (Preventive Medicine, Liverpool, 1957-60).

□ Total of new canine cases (1,700) presented for all complaints at Beaumont Animals' Hospital on first and third Saturdays of each month in 1954-59.

RESULTS

Incidence

The records of the 223 calculus cases from London have been examined for breed, sex and age incidence and the results are shown in Figs. 1 and 2. Data on the chemical composition of the calculi from these cases were not sufficient to allow an analysis for breed or site. The sample of 1,700 dogs presented at the Beaumont Animals' Hospital for all complaints has been reviewed for breed, sex and age and the results are given in the same figures. The incidence of calculi in those breeds which were represented by more than sixty dogs during the six-year period is shown in Table 1. The 223 cases of urolithiasis came from a total hospital population which was estimated at 11,049 new canine cases over the six year period. This figure was obtained from the sample population of 1,700 new cases

TABLE 1

BREED INCIDENCE OF CALCULI, LONDON, 1954-59

Breed	*No. of calculus cases*	*Estimated total dogs presented at clinic*	*Percentage incidence of calculi*
Dachshund	16	240	6·66
Cairn Terrier	5	78	6·41
Welsh Corgi	16	273	5.86
Scottish Terrier	23	448	5·13
Poodle (Miniature and toy)	18	494	3·65
Labrador Retriever	5	143	3·49
Cocker Spaniel	24	910	2·64
Pekingese	12	455	2·64
Wire Hair Fox Terrier ..	6	331	1·81
Yorkshire Terrier	2	136	1·47
Alsatian	6	769	0·78
Collie	1	214	0·47
Boxer	1	475	0·21
Bull Terrier	0	136	0·0
Chow	0	104	0·0
Other pure breeds	37	605	6·12
Mongrels	51	5,238	0·98
Total	223	11,049	2.02

presented at the clinic during the first and third Saturdays of each calendar month (144 Saturdays in all). It was found that one third of all new cases presented at the clinic arrived on Saturdays. The total new cases for the whole six years on all the Saturdays (312) would thus be 3,683 ($\frac{312}{144} \times 1{,}700$). The total number of new cases during the six years would then be three times this number i.e. 11,049. This estimated background population is the one against which the calculus cases have been viewed.

The relationship between the site of the calculi and the sex of the patients is shown in Table 2. Further information relating to breed, sex and age was obtained from the series of 122 stones examined in this laboratory. To facilitate comparison with the London figures the results are shown on the graphs (Figs. 1 and 2), and separately in Table 3.

TABLE 2
LONDON

Relationship between the site in which calculi were found and the sex of the animal

Site	*Male*	*Female*	*Not known*	*Total*
Urethra	52	0	0	52
Urethra and bladder.. ..	39	1	0	40
Bladder	36	82	1	119
Renal pelvis	0	7	0	7
Renal pelvis and bladder ..	0	4	1	5
Total	127	94	2	223

TABLE 3
LIVERPOOL

Relationship between the site in which calculi were found and the sex of the animal

Site	*Male*	*Female*	*Not known*	*Total*
Urethra	32 (31)	1 (0)	3	36 (31)
Urethra and bladder.. ..	8 (2)	1	0	9 (2)
Bladder	15 (5)	51 (35)	3 (25)	69 (65)
Renal pelvis	1	0 (1)	0	1 (1)
Renal pelvis and bladder ..	2	0 (2)	0	2 (2)
Unknown	2	2	1	5
Total	60 (38)	55 (38)	7 (25)	122 (101)

Bracketed figures are those of White (1944)

Composition

Some of our own collection of calculi were analysed by the method described by White (1944) and the rest (about 60) by the method of microchemical analysis described in this paper. A few stones have been examined in more detail but the results are not discussed here.

The stones have been classified according to their main constituents as phosphate (magnesium ammonium phosphate, calcium phosphate, or both), oxalate (calcium oxalate monohydrate, dihydrate, or both), cystine and urate (ammonium urate or uric acid) stones. Table 4 shows the relationship between the type of stone and the breed and sex incidence, while Table 5 gives the overall incidence of the stones in different sites in the urinary tract.

TABLE 4
LIVERPOOL

Relationship between the main chemical constituents of the calculi and the breed and sex of affected dogs

Breed	*Type of calculus*				
	Phosphate	*Oxalate*	*Cystine*	*Urate*	*Total*
Alsatian	1	0	2	0	3
Beagle	1	0	0	0	1
Boxer	1	1	0	1	3
Bull Terrier	2	0	0	0	2
Cairn Terrier	4	0	3	0	7 (3)
Chow	0	1	0	0	1
Cocker Spaniel	8	0	1	2	11 (5)
Collie	1	0	0	0	1
Dachshund	4	0	2	0	6
Dalmatian	0	0	0	9	9 (5)
Dandie Dinmont	2	0	0	0	2
Flat-coated Retriever	1	0	0	0	1
Fox Terrier	2	2	0	1	5 (7)
Golden Retriever	1	1	0	0	2 (1)
Griffon	0	1	0	0	1
King Ch. Sp.	2	0	0	0	2
Labrador Retriever	1	0	0	0	1
Lakeland Terrier	0	0	0	1	1
Norwich Terrier	2	0	0	0	2
Pekingese	6	4	0	0	10 (9)
Poodle (Miniature)	4	1	0	0	5
Scottish Terrier	7	0	0	0	7 (8)
Tibetan Spaniel	0	0	1	0	1
Welsh Corgi	8	2	2	0	12 (3)
West Highland Terrier	2	0	0	0	2 (1)
Yorkshire Terrier	1	0	0	0	1
Mongrel	10	4	2	0	16 (11)
No information	4	2	1	0	7
Total	75	19	14	14	122 (103)
Percentage	61·5	15·5	11·5	11·5	
Male	16	17	13		60 (40)
Female	54	1	0		55 (38)
Sex not known	5	1	1		7 (25)

Bracketed figures are those of White (1944)

Sixteen of the calculi of the Liverpool series have been examined by X-ray diffraction. The results (Table 6) confirm those of chemical

TABLE 5
LIVERPOOL

Relationship between the main chemical constituents of the calculi and the site in which they were found

Site	*Main constituent*				
	Phosphate	*Oxalate*	*Cystine*	*Urate*	*Total*
Urethra	6 (7)	12 (4)	8 (17)	10 (3)	36 (31)
Urethra and bladder ..	5	1 (2)	1	2	9 (2)
Bladder	60 (56)	5 (4)	3 (2)	1 (3)	69 (65)
Renal pelvis	0	0	0	1 (1)	1 (1)
Renal pelvis and bladder	0 (2)	0	2	0	2 (2)
Not known	4	1	0	0	5
Total	75 (65)	19 (10)	14 (19)	14 (7)	122 (101)

Bracketed figures are those of White (1944)

TABLE 6
ANALYSIS OF CALCULI BY X-RAY DIFFRACTION

X-ray crystallographic analysis	*Microchemical analysis*	
	Major constituents	*Minor constituents*
Phosphate stones		
Struvite ($MgNH_4PO_4,6H_2O$) + trace of appatite ($Ca_{10}(PO_4)6(OH)_2$)	Triple phosphate ($MgNH_4PO_4,6H_2O$)	Calcium phosphate
Struvite	Triple phosphate	Calcium phosphate
Struvite + trace of apatite	Triple phosphate	Calcium phosphate
Struvite + trace of apatite	Triple phosphate + calcium phosphate	Calcium oxalate
Struvite	Triple phosphate	Calcium phosphate
Struvite	Triple phosphate	—
Apatite	Calcium phosphate	Triple phosphate + calcium oxalate
Oxalate stones		
Weddellite $CaC_2O_4,2H_2O$	Calcium oxalate	Calcium phosphate
Whewellite CaC_2O_4,H_2O	Calcium oxalate	Calcium phosphate
Whewellite in *centre*, struvite at *periphery*	Calcium oxalate	Calcium phosphate
	Triple phosphate + calcium phosphate	Calcium oxalate
Weddellite	Calcium oxalate	Calcium phosphate
Whewellite	Calcium oxalate + urate	Calcium phosphate
Cystine stones		
Cystine	Cystine	Phosphate
Urate stones		
Uric acid	Uric acid or urate	—
Urate (no match in ASTM index)	Uric acid or urate	—
Urate (no match in ASTM Index)	Uric acid or urate	Phosphate

analysis and provide evidence of the precise crystalline substance which forms the main constituent of the stones. Organic matter would not be identified, and minor constituents would be missed unless the diffraction pattern were pronounced. We are not aware of the previous use of this method for calculi from dogs, but a number of workers have used it to study human stones and have obtained valuable information about their structure and composition.

DISCUSSION

Krabbe (1949) in Copenhagen, and Krook and Arwedsson (1956) in Stockholm both quote the incidence of urolithiasis as 0.6 per cent of the total cases treated in their clinics. If the estimated number of dogs coming to the Beaumont Animals' Hospital over the years 1954-1959 is taken as 11,049 the incidence of urinary calculi there is approximately 2.0 per cent.

The breed incidence for the London cases shows that calculi are particularly common in the Dachshund, Cairn, Corgi and Scottie. The incidence is between 5.13 and 6.66 per cent of the animals of these breeds coming to the clinic. The figures for the Liverpool series confirm that calculi are common in these four breeds. The incidence of calculi is also above average in the London figures for the Poodle, Labrador Retriever, Cocker Spaniel and Pekingese. In the Liverpool series there was only one calculus case among Labradors, but the other three breeds were well represented. The incidence was under one per cent in five breeds—Alsatian, Collie, Boxer, Bull Terrier and Chow. The numbers of Alsatians and Boxers presented were sufficiently high to make it likely that the low incidence in these breeds is a real one. The numbers of calculus cases were also low for all these breeds in the Liverpool series.

A word of comment is necessary regarding the apparently high incidence (6.12 per cent) in "other pure breeds". The reason for this is the sampling error in these breeds, very few of which appeared in 1,700 dogs taken from the London records. An extreme example is the Irish terrier in which there were five calculus cases over the six years but no animals of this breed happened to be presented on the Saturdays when the sample of the population was determined. Among such poorly represented breeds there may well be some in which urinary calculi are very common e.g. Irish Terrier, Sealyham, Pomeranian and Dalmatian. It is not possible to quote figures for these breeds, however, because the total numbers are so small. It would be surprising if the incidence in the Dalmatian, for instance, was not at least as high as in most other breeds. Nine calculus cases were encountered in this breed in Liverpool, which suggests a high incidence, but there were only two cases in London among an estimated background population of 39. It is unsafe to quote figures for incidence from such small numbers. The incidence of calculi in mongrels was only about half the average incidence for all dogs.

These findings on breed incidence agree with those of Krabbe (1949), Krook and Arwedsson (1956) and Brodey (1955). Detailed comparison with the figures from Vienna (Uller, 1959) is difficult because there is little indication of the relative popularity of the different breeds there.

It might be thought that the population of dogs sent to the London Clinic would be very different in breed incidence from that in the country as a whole, but comparison with the figures of Kennel Club registrations for the corresponding years shows, with a few exceptions, a striking agreement. There are two possible reasons for the exceptions. Dalmatians and Chows, for instance, are much more common in the clinic population than in the Kennel Club figures. This might be because there are relatively more of them in the population from which the clinic draws its cases or because of a high incidence of one or more diseases which necessitate treatment. In contrast, mongrels only slightly outnumber pure breeds in the London population, whereas there are many times as many of them in the country as there are pure breeds. It may be that mongrels are less often ill or that they are less often brought to clinics when they are. It is unlikely that there are so few of them as the clinic records suggest. Unfortunately, little is known about the structure of the dog population and the diseases which occur in the different breeds. Further analyses of clinic populations for this purpose would be valuable.

The breed incidence recorded in this paper is set against a background of sick dogs, not healthy ones. The relationship which the clinic population bears to the total dog population is not known, and it certainly varies from one clinic to another. Apart from the Dalmatian, there is no evidence of any particular breed character which favours calculus formation, except for the work of Krook and Arwedsson (1956) which suggests that chondrodystrophic breeds are more likely to become affected. Their statistics from Stockholm, where all dogs are registered, show a higher incidence of calculi in the Boston Terrier, Dachshund, Pekingese and French Bulldog, which were all earlier classed as chondrodystrophic breeds by Hansen (1952) and were shown to be characterised by a disturbance of endochondral ossification, giving rise to disproportionate dwarfism, and a tendency towards disc protrusion. It is certainly possible that a disturbance of calcium and phosphorus metabolism in any particular breed might favour the formation of phosphate and oxalate stones, and might even have an effect on cystine stones as well if the solubility of these substances is influenced by Ca and P in the urine. It is doubtful, however, whether this is likely to be the main factor causing the high incidence in all four breeds in which we found calculi to be most common.

Another possible factor is confinement. Small dogs are more often kept confined to the house or flat, while large dogs like Alsatians and Boxers are given more freedom. Mongrels may be

left to roam more often than pure breeds. Confinement may act by favouring urinary retention and so predisposing to primary calculi directly and to secondary ones by making infection of the bladder more likely.

It has for long been known that the Dalmatian has a very high level of uric acid in its urine and so is predisposed to the formation of urate stones. The incidence of urate calculi in Dalmatians in our own series approaches that of stones of all types in the other breeds which have a high incidence of urolithiasis. All nine of the Liverpool stones from Dalmatians were composed of urate, and more than half of all the urate stones examined came from animals of this breed. Other breeds than the Dalmatian can produce urate calculi, but much less commonly. With the exception of the Dalmatian, no breed seems to be especially prone to any particular type of stone.

Among the eleven cystine stones recorded by Uller (1959), nine were in Dachshunds. Our corresponding figure is two out of 14 cystine cases and it may be that the high incidence among Dachshunds in Vienna is partly due to the popularity of this breed in the area.

Uller's figures for the relative incidence of the four chemical types of calculi show that of 163 stones he analysed 65 per cent were phosphate, 20.9 per cent oxalate, 6.8 per cent cystine and 7.3 per cent urate. These are in good agreement with the findings reported here from Liverpool where of the 122 stones 61.5 per cent were phosphate, 15.5 per cent oxalate, 11.5 per cent cystine and 11.5 per cent urate. In 1944, White found cystine stones to be more common than oxalate stones—of 103 stones 63.1 per cent were phosphate, 10.7 per cent oxalate, 18.5 per cent cystine, and 7.7 per cent urate. The American material of Brodey (1955) contained no oxalate or cystine stones, whereas Bloom (1960) lists cystine stones as the second most common type of stone (presumably in the United States). We have ourselves identified cystine stones in material from the U.S.A. and there is no reason to think that the incidence of cystinuria and cystine stones differs from what it is in Great Britain. It was indeed in the United States that the classical study of canine cystinuria in Irish Terriers was made by Brand and his co-workers more than twenty years ago. We have had no difficulty in finding dogs with cystinuria by tracing back cases of cystine stones sent for analysis from practitioners and clinics.

All the Liverpool cases of urate and cystine stones have been in male dogs, and 17 of 18 cases of oxalate stones were also in the male. All the cystine stones in the London series were in males (16), like those in our own series of 1944 (19) and 1960 (14)—a total of 49 cases. This makes it likely that cystine calculi are in fact confined to the male sex. Brand and Cahill (1940) mentioned a single female cystinuric Irish Terrier in their analysis of animals bred from cystinuric parents, but we have not been able to confirm the accuracy of this record.

Urate sometimes precipitates out in the bladder as a loosely bound, crumbling mass which contains also appreciable amounts of phosphate and occasionally traces of oxalate also. The only urate stone encountered in a bitch was of this kind and was found in a Dalmatian in the London series. It was a mixed stone, containing phosphate and carbonate as well as urate. This type of stone may well form very rapidly and so cause symptoms in the bitch, as compared with the more usual small, smooth, spherical urate stones which only cause trouble when they obstruct the urethra of the male as it enters the groove of the os penis. While urate calculi are thus not entirely confined to the male dogs it is in this sex that they nearly always produce their clinical effects.

The large size of the calculi, whether single or multiple, is a feature of phosphate urolithiasis. Why phosphate stones form such a high proportion of the calculi in the bitch, as compared with the male dog, is not known. One possible explanation is that infection plays a part in their development and is more common in the female. Another explanation is that, of the four chemical types of stone in dogs, only phosphate stones commonly reach a large size or accumulate to form a large mass of calculus material. Stones up to 1 cm. in diameter are readily passed through the female urethra and so most urate or oxalate stones would be too small to cause obstruction in the bitch. This is borne out by the figures which show that whereas the main cause of urinary symptoms in male dogs is blocking of the urethra with small stones of all four chemical types, the main cause in the bitch is vesical distension or urethral obstruction by large single phosphate stones or accumulations of smaller ones. Thus 54 of 70 cases of phosphate stones were confined to the bladder. These two possible factors of infection and size of stone may both be concerned in causing the high incidence of phosphate stones in bitches. The first may favour the female sex for their development, while the second may account for their ability to cause obstruction.

The occasional finding of relatively small oxalate stones causing symptoms in the bitch is probably due to the sharp crystalline plates composed of calcium oxalate dihydrate (weddellite) which project from many oxalate stones. Such stones may cause serious cystitis in the bitch with extensive haemorrhage, even when the stones are quite small.

The data on the age incidence of calculus cases shows that whereas puppies under one year are the most frequent age group seen at the London clinic, urolithiasis at such an early age is very uncommon. Only one case, a five months male English Setter, was encountered and it had a large vesical phosphate stone. Three cases in dogs under a year old occurred in the Liverpool series. The peak incidence for calculi in the London records was in the six year age group, but there is a high incidence in all age groups from six to ten years, except for the seven year group. In the Liverpool series the peak incidence is at 4 to 7 years, which is the same as in White's

1944 series. Calculi might well be common in still older dogs if it were not for the fact that many recurring cases will have died after two or three years, sometimes after repeated cystotomies or urethrotomies. The average age incidence for calculi in the London series is 6.5 years, as compared with 4.7 years for the average age for all conditions seen at the clinic. The Liverpool average for calculus cases is 5.9 years. Urolithiasis is thus most common between the ages of 4 and 10 years, with a peak at 6 to 7 years. These figures agree well with those of Krook and Arwedsson (1956) Brodey (1955) and Uller (1959), for three different countries.

Both the London and the Liverpool figures show renal calculus formation to be rare in both sexes. This is in marked contrast to the high relative incidence of renal stones in man where they account for more than half of all urinary calculi. The low incidence in the dog may be partly explained by the better orientation of the canine kidney with respect to drainage of the simple renal pelvis, so that stasis is prevented unless there is serious obstruction lower down the urinary tract. No cases of ureteral calculi were encountered in the dog, whereas they are quite common in man. The striking difference in the site incidence in the two sexes in dogs is probably explained mainly by the anatomical differences between the urethra in the male and female.

The results of X-ray crystallographic analysis confirmed those obtained by microchemical analysis and also gave evidence of the exact crystalline form of the main constituents. They identified calcium oxalate as the dihydrate (weddellite) or the monohydrate (whewellite), and calcium phosphate as apatite. Usually, however, the method failed to identify the minor constituents which microchemical analysis revealed. In the case of urate stones, the diffraction pattern was often difficult to identify with standard A.S.T.M. reference cards. In this connection Jensen (1940) mentioned that the uric acid diffraction pattern depended on the treatment of the crystals prior to analysis.

Prophylaxis requires only that the main constituents of calculi are known; a knowledge of the exact crystalline form is probably unnecessary for this purpose. X-ray diffraction is tedious and expensive and is unlikely to provide a practicable alternative to chemical analysis for routine use, but it can give valuable information in studies on the method of formation of calculi and the relationships of their major and minor components. The same applies to the study of thin sections of calculi, which reveal the nature and orientation of the crystalline components and their relationship with the organic matrix. Our preliminary observations suggest that X-ray diffraction and studies of thin ground sections and stone fragments in polarized light are valuable supplementary methods in the study of calculi in dogs. They have already been used with human calculi.

It is probable that the four different chemical types of stone have

different aetiologies, but the factors responsible for any of them are as yet imperfectly understood. The possible factors concerned include the total daily excretion of the stone-forming constituents in the urine (e.g. cystine in cystinuria, uric acid in Dalmatians), the urinary pH and concentration, the presence of stasis and bacterial infection, and the levels of a variety of ions and of urinary colloids. Until more is known about the precise factors concerned for each type of stone there is little that can be done to prevent calculi or to reduce the likelihood of their recurrence.

General measures to prevent recurrence comprise controlling infection when this is present, increasing fluid intake all round the clock in order to keep the urine as dilute as possible, and encouraging regular emptying of the bladder. Where the calculus constituent is mainly exogenous it may be possible to reduce the dietary intake. When the stone-forming substance is precipitated in acid urine and is soluble in alkaline urine (cystine and uric acid) it may be useful to raise the urinary pH by daily doses of sodium bicarbonate or sodium citrate. Changes in pH will have little effect on the formation of oxalate stones. The urine should be kept acid when phosphate stones have been identified. These differences emphasise the importance of identifying the type of stone present and also the need for further research into the conditions which favour or inhibit the formation of calculi. Our own work is continuing with two types of stone, cystine and urate, and will form the subject of later papers.

CONCLUSIONS

A method of qualitative microchemical analysis suitable for urinary calculi is described. X-ray diffraction was used in the analysis of a small series of stones and the results are compared with those of chemical analysis.

Figures from the Beaumont Animals' Hospital, Royal Veterinary College, London, indicate an overall incidence of calculi in the years 1954-1959 of 2.0 per cent (223 cases among an estimated population of 11,049 dogs presented at the clinic for all diseases).

Chemical analysis of calculi from 122 dogs in different parts of England, Scotland and Wales shows that 61.5 per cent were composed mainly of phosphate, 15.5 per cent of oxalate, 11.5 per cent of urate and 11.5 per cent of cystine.

Neither sex is more prone than the other to urolithiasis, but whereas the main cause of symptoms in the male dog is the presence of small stones of urate, oxalate or cystine which cause obstruction of the urethra where it enters the groove in the os penis, in the bitch cystitis of varying severity results from the presence in the bladder of phosphate calculi. Renal calculi are rare in the dog.

On present evidence it is not possible to say with certainty that cystine stones are confined to the male dog, though it is very likely that this is so since all 49 so far encountered have been in this sex. Oxalate and urate stones occur in both sexes but most often cause

clinical symptoms in the male, because they are usually too small to obstruct the urethra of the bitch.

Figures from the London and the Liverpool Veterinary Schools, respectively, show that several household breeds are especially prone to calculi, particularly the Dachshund, Cairn, Welsh Corgi and Scottish Terrier (5.1 to 6.7 per cent). The incidence is low in the Alsatian, Collie, Bull Terrier, Boxer and Chow (0.0 to 0.8 per cent). The incidence in mongrels was 0.98 per cent.

Calculi in Dalmatians are mainly urate stones, but urate calculi are not confined to this breed.

Urolithiasis is most common between the ages of four and ten years, with a peak incidence at 6 to 7 years. It is rare in puppies.

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